

**THE EFFECTS OF THERAPEUTIC HYPOTHERMIA ON CYTOCHROME P450-
MEDIATED METABOLISM: STUDIES IN TRANSLATIONAL RESEARCH**

by

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This work is dedicated to Fernando Fusco

Thanks Pop

THE EFFECTS OF THERAPEUTIC HYPOTHERMIA ON CYTOCHROME P450-MEDIATED METABOLISM: STUDIES IN TRANSLATIONAL RESEARCH

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University of Pittsburgh, 2007

Therapeutic hypothermia decreases neurological damage in patients experiencing out-of-hospital cardiac arrest (CA). In addition to hypothermia, critically ill patients are treated with an extensive pharmacotherapeutic regimen. The majority of these medications are hepatically eliminated via the cytochrome P450 (CYP450) system. Changes in drug clearance could limit the putative benefit of hypothermic therapy. With the increased use of therapeutic hypothermia and the fact that critically ill patients receive multiple medications, it is crucial to understand the effects of hypothermia on the disposition and metabolism of drugs used in this population. Thus, it was the overall aim of this research to investigate the effects of therapeutic hypothermia on CYP450-mediated metabolism in an animal model of CA, in human liver microsomes, and in normal healthy subjects. Specifically, hypothermia produced a ~2 fold decrease in the systemic clearance (Cl_s) of intravenous chlorzoxazone, a specific CYP2E1 probe substrate, in a CA rat model when compared to CA rats treated under normothermic temperatures. The mechanism behind this decrease in Cl_s was a hypothermia-mediated decrease in the affinity of CYP2E1 for chlorzoxazone. We extended the experimental period to investigate the effects of hypothermia after re-warming, on CYP2E1 and CYP3A2 activity and expression. Our results indicate that rats with CA treated under normothermic temperatures demonstrated a significant decrease in the

activities of CYP2E1 and CYP3A2, 24 hrs after injury compared to control. Furthermore, CA significantly decreased the expression of *CYP3A2*, but not the expression of *CYP2E1*. CA also produced a ~ 10-fold increase in plasma concentrations of interleukin-6 (IL-6) compared to Control. The CA-mediated reduction in CYP3A2 and CYP2E1 activity, mRNA, and the increase in IL-6 plasma concentrations was attenuated by hypothermia. We also investigated the effects of mild and moderate hypothermia on CYP2E1 and CYP3A4 enzyme kinetics in human liver microsomes. Both mild and moderate hypothermia significantly decreased the V_{max} of CYP2E1 and CYP3A4. However, hypothermia increased the K_m of CYP2E1 but not CYP3A4. These data demonstrate that mild and moderate hypothermia may produce isoform specific alterations of human CYP450-mediated metabolism. Lastly, in a pilot analysis, we showed that mild hypothermia may potentially alter the Cl_s and the volume of distribution (V_{ss}) of midazolam in mildly hypothermic normal healthy volunteers.

Collectively, this work provides evidence that therapeutic hypothermia alters CYP450-mediated metabolism both during cooling and after re-warming. Based on the magnitude of these changes it is clear that intensivists should be cognizant of these alterations and monitor drug levels and outcomes in their patients when possible. In addition to increased clinical attention, future research efforts are essential to delineate precise dosing guidelines and mechanisms of the effects of hypothermia on drug disposition, metabolism, and response.

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PREFACE

Over the past 11 years, I have been a student here at the University of Pittsburgh, which includes undergraduate, professional, and graduate school. There have been numerous people who have helped me in my journey to achieve one of my greatest goals; attainment of the degree of Doctor of Philosophy in the Pharmaceutical Sciences. They include professors, mentors, coaches, friends, and family. It is difficult to thank everyone who has contributed to my success; however there are a few people who have played a major role in my achievements. I would first like to thank my advisor Dr. Samuel Poloyac, who I have had the privilege of working for and learning from since 2002. His mentorship has taught me about both science and life. If it were not for his enthusiasm and dedication to the work, I would not have been where I am today. I would also like to thank my co-advisor Dr. Patrick Kochanek. Dr. Kochanek's mentorship has been critical to the development of this work as well as the development of my skills as a scientist. I would like to thank the rest of my committee including Dr. Robert Gibbs, Dr. Raman Venkataramanan, Dr. Regis Vollmer, and Dr. Michael Zemaitis, for their support, tutelage, and guidance over the past 4 years. I would like to thank Dr. Robert Bies for his help, support, and belief in my abilities as a scientist and a person. Lastly, I would like to thank Dr. Dave Hostler and Dr. Clifton Callaway for their help and dedication to the clinical aspects of this work.

My decision to attend graduate school was made with the support of my family and friends. I will first begin by thanking some of the people who have provided me a family away

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ABBREVIATIONS

AHA: American Heart Association
ANOVA: analysis of variance
AP-1: activating protein-1
APR: acute phase response
AUC: area under the plasma-concentration time curve
BIS: bispectral index
BMI: Body Mass Index
CA: cardiac arrest
C/EBP alpha: CCAAT/enhancer binding protein alpha
Cl_{int}: intrinsic clearance
CL_H: hepatic clearance
CPB: cardiopulmonary bypass
CL_R: renal clearance
CL_S: systemic clearance
CYP450: cytochrome-P450
CZN: chlorzoxazone
6-OH CZN: 6-hydroxychlorzoxazone
DHCPB: deep hypothermia cardiopulmonary bypass
EEG: electroencephalograph
ELISA: enzyme linked immunosorbent assay
FAD: flavin adenine dinucleotide
FDA: Food and Drug Administration
FMN: flavin mononucleotide
Fu: fraction unbound
GCS: Glasgow Coma Score
GFR: glomerular filtration rate
HNF: hepatocyte nuclear factor
HPLC: high performance liquid chromatography
ICP: intracranial pressure

ICU: intensive care unit
IL-1 β : interleukin -1 β
IL-6: interleukin-6
ILCOR: International Liaison Committee on Resuscitation
K: elimination rate constant
K_m: Michaelis-menten constant
LPS: lipopolysaccharide
LOQ: limit of quantification
MABP: mean arterial blood pressure
MAC: mean alveolar concentration
MDZ: midazolam
1-OH MDZ: 1 -hydroxymidazolam
4-OH MDZ: 4 -hydroxymidazolam
NADPH: nicotinamide adenine dinucleotide
NO: nitric oxide
NO₂: nitrite
NO₃: nitrate
ROSC: return of spontaneous circulation
t_{1/2}: half-life
TBI: traumatic brain injury
TNF- α : tumor necrosis factor - α
TST: testosterone
6 β -OH TST: 6 β -hydroxytestosterone
11 β -OH TST: 11 β -hydroxytestosterone
UGT: UDP-glucuronsyltransferase
V_d: volume of distribution
V_{ss}: volume of distribution at steady-state
V_{max}: maximal velocity

1.0 CHAPTER 1

[Tortorici MA, Kochanek PM, and Poloyac SM. Effects of Hypothermia on Drug Disposition, Metabolism, and Response: A Focus of Hypothermia-Mediated Alterations on the Cytochrome P450 Enzyme System *Crit Care Med.* 2006, Under Review]

INTRODUCTION AND BACKGROUND

Determining the effects of a new chemical entity on the disposition and metabolism of other drugs is an essential component in the new drug approval process according to the Food and Drug Administration (FDA) (FDA 1997). For non-pharmacological therapies implemented into clinical practice, the same stringency of investigation is not as clear. Frequently, non-pharmacological therapies, such as therapeutic hypothermia, are clinically utilized with limited understanding of the effects on the disposition and metabolism of the most commonly used drugs in patients. This creates the potential for therapy-drug interactions.

Therapeutic hypothermia has long been used in cardiac surgery and as a second tier therapy for refractory raised intracranial pressure (ICP) in traumatic brain injury (TBI) (Clifton, Allen et al. 1993; Tonz, Mihaljevic et al. 1995). Recently, mild and moderate hypothermia has demonstrated benefit in both adults and infants experiencing cardiac arrest (CA) and may have some application in TBI and stroke, which has greatly expanded the scope of this therapy in the intensive care unit (ICU) setting (Clifton, Allen et al. 1993; Tonz, Mihaljevic et al. 1995; Clifton, Miller et al. 2001; Bernard, Gray et al. 2002; Group 2002; Dalton Dietrich and Kuluz 2003; Samson, Nadkarni et al. 2006). In the CA population, therapeutic hypothermia has been shown to decrease secondary neurological damage (Bernard, Gray et al. 2002; HACSG 2002). Data in TBI and stroke has also been promising and has begun to increase the role of therapeutic hypothermia as a potential therapy (Clifton, Allen et al. 1993; Marion, Penrod et al. 1997;

Clifton, Miller et al. 2001; Berger, Schabitz et al. 2002; Dalton Dietrich and Kuluz 2003; De Georgia, Krieger et al. 2004). Hypothermia in surgery requiring CPB has been used to decrease the underlying inflammatory reaction due to cellular activation by the material of the extracorporeal circuit and the surgical trauma (Westaby 1987; Tonz, Mihaljevic et al. 1995). The evidence provided in these studies has led to an increase in the clinical implementation of hypothermia and has spurred ongoing trials to determine the optimal depth, duration, and re-warming rate required for full benefit of this intervention.

With the increased use of therapeutic hypothermia and the fact that critically ill patients are subjected to an extensive pharmacotherapeutic regimen, there is an increased probability that alterations in drug concentrations will occur during treatment. These alterations can result in toxic or sub-therapeutic dosing, thereby, limiting the optimal application of hypothermia. Therefore, it is important for the intensivist to understand the effects of therapeutic hypothermia on the disposition of drugs as a guide for dosing patients being treated with therapeutic hypothermia. As depicted in **Figure 1**, the effects of hypothermia on drug disposition are a complex interplay of two major effects, during cooling and the effects post re-warming. As will be evident throughout this dissertation, the hepatic metabolism of certain drugs is decreased during cooling, more specifically CYP450-mediated metabolism. This suggests that intensivists should decrease the dose of drugs that rely on CYP450-mediated metabolism in order to achieve optimal plasma concentrations. However, studies have shown that the potency and efficacy of certain drugs is decreased during cooling, suggesting the potential need for increased dosage to achieve an optimal response. Hypothermia also produces regulatory effects by mitigating cellular damage caused by injury and returning drug metabolism to normal. It is the purpose of this introductory chapter to discuss the known effects of hypothermia on both drug disposition

and response during the acute and chronic phase to set the foundation for the studies presented in the following chapters of this dissertation. Specifically, we have focused this chapter on drugs commonly employed in critical care that are eliminated via CYP450-mediated metabolism.

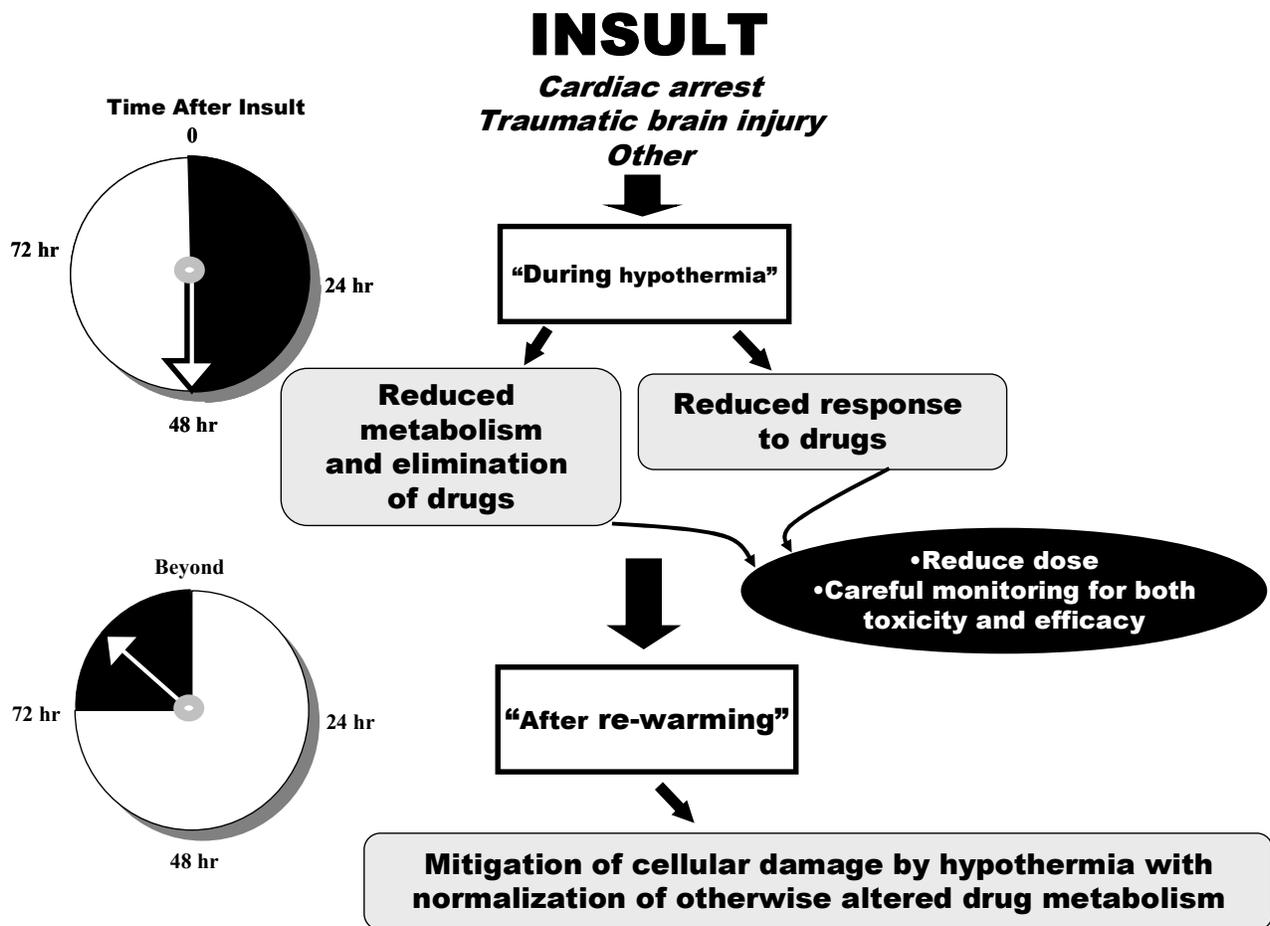


Figure 1: The effects of therapeutic hypothermia on drug disposition and response

The acute effects (during cooling ranging from 12-48 hours) are shown to decrease metabolism and response. Intensivists should monitor for toxicity and response and appropriately change the dose of drugs accordingly. The chronic effects (after re-warming) result in the mitigation of cellular damage by hypothermia with normalization of otherwise altered drug metabolism.

1.1 THERAPEUTIC HYPOTHERMIA

Clinical neurological injury caused by insults such as CA and TBI, contributes significantly to the morbidity and mortality of patients and is a major burden on health care in the United States (Narayan, Michel et al. 2002; Rea, Pearce et al. 2004; De Keyser, Uyttenboogaart et al. 2005). Reports have shown that approximately 200,000-500,000 patients experience an out-of-hospital CA each year (Rea, Pearce et al. 2004). Traumatic brain injury accounts for 500,000 cases annually in the United States (Doppenberg and Bullock 1997). The common cause of morbidity in survivors of these insults is secondary neurological damage (Levy, Caronna et al. 1985; Jorgensen and Holm 1998). Drug therapy has been largely supportive and has not targeted this secondary neurological damage (Laver, Farrow et al. 2004).

Therapeutic hypothermia decreases multiple pathological processes which occur after clinical neurological injury (Karibe, Chen et al. 1994; Lei, Tan et al. 1994; Aibiki, Maekawa et al. 1999; Bernard, Gray et al. 2002; Group 2002). The Advanced Life Support Task Force of the International Liaison Committee on Resuscitation (ILCOR) and recently the European Resuscitation Council has recommended mild hypothermia for unconscious adult patients with restoration of spontaneous circulation (ROSC) following out-of-hospital cardiac arrest due to ventricular fibrillation and the AHA Advisory Committee for CRR suggested it as class IIa recommendation (Nolan, Morley et al. 2003). This recommendation is based on the findings of two landmark clinical trials reporting neurological benefit of hypothermia in patients experiencing out-of-hospital cardiac arrest (Bernard, Gray et al. 2002; Group 2002). A multi-center trial conducted by The Hypothermia After Cardiac Arrest Study group, investigated the effects of mild hypothermia on neurological outcome and mortality in 273 patients resuscitated after CA due to ventricular fibrillation (Group 2002). Patients treated with mild hypothermia

had a more favorable neurological outcome compared to normothermic patients, six-month post-CA (Risk Ratio: 1.4, $p < 0.009$) Furthermore, mortality was decreased in the hypothermia group compared to the normothermia group, (Risk Ratio; 0.74 (confidence interval = 0.58-0.95)). Bernard *et al* showed that mild hypothermia improved cardiovascular effects in survivors of out-of-hospital CA (Bernard and Buist 2003). This study evaluated 77 patients who experienced out-of-hospital CA and were randomized to receive 12 hours of mild hypothermia (32°C) or standard supportive care (37°C). Results demonstrated 49% of patients treated with hypothermia were discharged to home or a rehabilitation facility, as compared to 26% of those not treated with hypothermia, ($p=0.046$). Germaine to this dissertation, the temperatures shown to be efficacious in these studies were between 32°C to 34°C, and were applied for 12 to 24 hrs. Recently, mild hypothermia (34°C) applied for 72 hours was shown to improve long-term neurological outcome in perinatal asphyxia, expanding the use of this novel therapy (Shankaran, Laptok et al. 2005).

Use of hypothermia in TBI has demonstrated benefit in patients with elevated intracranial pressure (ICP). Marion *et al.* showed that TBI patients with Glasgow coma scale (GCS) scores of 5 to 7, who were treated with hypothermia (32°C to 33°C) had improved neurological outcomes compared to patients maintained under normothermic conditions (Marion, Penrod et al. 1997). However, Clifton *et al.* failed to show a beneficial effect of moderate hypothermia (33°C) compared to normothermic management in patients with TBI (Clifton, Miller et al. 2001). Efficacy of mild or moderate hypothermia in TBI and other traumas as a secondary therapy for control of refractory raised ICP remains controversial (Kochanek and Safar 2003). Sources of variance that have been identified include the pathophysiologic mechanisms, secondary insults, and stress associated with other non-pharmacological therapies Relevant to this dissertation, one source of variance identified as a potential confounder is the use of pharmacological agents such

as sedatives, narcotics, and neuromuscular blockers during hypothermia, which may produce pharmacologic interactions. Changes in drug clearance due to hypothermic therapy could lead to a key toxicity which may limit the putative benefit of this novel therapy. With an increase in clinical implementation of hypothermia, changes in drug disposition could result in significant adverse drug events in ICU patients.

1.2 ADVERSE DRUG EVENTS IN CRITICAL CARE MEDICINE

In addition to non-pharmacological measures, such as hypothermia, patients experiencing critical care insults are subjected to an extensive pharmacotherapeutic regimen (Cullen, Sweitzer et al. 1997). The approach to therapy in this patient population is divided into multiple phases including support in the acute phase, measures to mitigate the pathologic process, and chronic administration during rehabilitation (Bradberry and Fagan 2002; Stringer and Lopez 2002). **Table 1** lists the most commonly used medications in the critical care population along with their major metabolic routes of elimination. The most commonly employed drug classes in this patient population include anti-arrhythmics, neuromuscular blockers, sedatives/hypnotics, anesthetics, vasodilators, and anti-coagulants. Since critical care patients are subjected to a myriad of drug therapy, they are the prime candidates for altered drug disposition, metabolism and response as a result of hypothermia.

Cullen *et al* showed that adverse drug events in ICU patients are nearly two-fold higher than in patients on general care units (Cullen, Sweitzer et al. 1997). They also stratified adverse drug events based on drug class and determined that significant differences in adverse events between ICU and non-ICU patients were observed for sedatives, cardiovascular drugs, anti-

arrhythmic drugs, muscle relaxant, and anticoagulant medications. Kopp *et al*, performed a study investigating medication errors and adverse drug events in the ICU (Kopp, Erstad et al. 2006). They found that drug interactions comprised one of the major categories of medication errors in critically ill patients in the ICU. Pharmacokinetic drug interactions include interactions affecting the absorption, distribution, metabolism, and excretion of drugs. Furthermore, TBI patients are exposed to a greater number of pharmacotherapeutic agents as compared to CA patients. This creates the potential for increased probability for drug-therapy interactions in TBI patients and may account for some of the variance in benefit observed with the application of hypothermia after TBI. The most clinically relevant pharmacokinetic drug interactions are those which affect the metabolism and excretion (both renal and biliary) of a drug. Multiple drug interactions which occur in the ICU are due to changes in the metabolism and excretion of drugs, specifically involving the CYP450 enzyme system (Boucher and Hanes 1998; Pea and Furlanut 2001; Krishnan and Murray 2003). Therefore, we can conclude that the addition of therapeutic hypothermia into clinical practice in the ICU could increase the probability of therapy-drug interactions due to the temperature effects on CYP450 functionality. Based on the importance of the CYP450 enzyme system in the disposition of multiple clinically relevant and potentially toxic medications, the focus of this work will be on the CYP450 system.

1.3 CYTOCHROME P450 ENZYMES

Cytochrome P450 enzymes are a ubiquitous super-family of monooxygenases which are critical in the metabolism of a wide variety of xenobiotics and endobiotics (Guengerich 1991; Anzenbacher and Anzenbacherova 2001). In 1958, Klingenberg demonstrated that when rat

Table 1: Commonly used ICU medications and their pathway of elimination

Drug Class	Medication	Elimination Pathway
Antiarrhythmics	Amiodarone	CYP3A4
	Bretylium	Excreted unchanged
	Procainamide	N-acetyltransferase
	Lidocaine	CYP1A2 & CYP3A4
	Sotalol	Renal Excretion
Beta-blockers	Metoprolol	CYP2D6 & CYP2C9
	Atenolol	Renal Excretion
Inotropic agents	Digoxin	CYP3A4
Calcium channel blockers	Verapamil	CYP3A4
	Diltiazem	CYP3A4
	Amlodipine	CYP3A4
	Nifedipine	CYP3A4
Benzodiazepines	Midazolam	CYP3A4
	Alprazolam	CYP3A4
Anesthetics	Propofol	CYP2B6
Opioids	Morphine	CYP2C & CYP3A
	Codeine & derivatives	CYP2C & CYP3A
	Fentanyl	CYP3A4
Anticonvulsants	Phenytoin	CYP2C9 & CYP2C19
	Carbamezipine	CYP3A4
Antimicrobials	Glycopeptides	Renal Excretion
	Aminoglycosides	Renal Excretion
	β -lactams	Renal Excretion
	Macrolides	CYP450s
	Fluroquinolones	CYP450s
	Rifampin	CYP3A4
	Tetracyclines	CYP450s
Proton pump inhibitors	Pantoprazole	CYP3A4 & CYP2C19
	Omeprazole	CYP3A4 & CYP2C20
	Lansoprazole	CYP3A4 & CYP2C21
H2-blockers	Famotidine	CYP450s
	Ranitidine	CYP450s
Corticosteroids	Methyprednisolone	CYP450s
	Prednisone	CYP450s
	Dexamethasone	CYP450s
Neuromusclar blockers	Vecuronium	CYP450s
	Atracurium	Renal Excretion

liver microsomes were incubated with carbon monoxide (CO) a peak at 450 nm was observed (Klingenberg 1958). This finding was unique in that heme proteins typically absorb light at 420 nm. In the early 1960s, the studies of Omura and Sato identified the peak at 450 nm as a hemoprotein they named cytochrome P450 (Omura and Sato 1962; Omura and Sato 1964). In addition, Mason *et al* demonstrated that CYP450 was responsible for incorporation of one oxygen atom into progesterone, describing this CYP450 enzyme as a monooxygenase enzyme (Mason, North et al. 1965).

CYP450 enzymes perform critical functions in multiple forms of life including vertebrates, invertebrates, fungi, plants, as well as prokaryotic organisms. CYP450 enzymes play key roles in steroid hormone biosynthesis, the activation and detoxification of many drugs and environmentally contaminating chemicals, the metabolism of polyunsaturated fatty acids (such as arachidonic acid), activation of vitamins A and D3 to biologically active hormones, the synthesis of a vast array of secondary metabolites in plants and insects and the metabolism of contaminating environmental chemicals to toxic and carcinogenic agents (Estabrook 2003). This wide range of physiological functions is due to the ability of CYP450 enzymes to metabolize various substrates.

The ability of CYP450 enzyme to metabolize multiple substrates is due to the mechanism of catalytic activity (Gonzalez 1997). The CYP450 enzyme is comprised of an apoprotein coat and a protoporphyrin IX heme moiety as depicted **Figure 2A**. The heme protoporphyrin IX is non-covalently bound to the P450 protein and is the same heme protein found in hemoglobin, myoglobin, cytochrome c, and other enzymes such as xanthine oxidase and myeloperoxidase. The heme is covalently bound through a thiol linkage to a cysteine residue on the CYP450 protein via its complexation with molecular iron. The apoprotein coat and the heme iron form a

functional P450 enzyme with the iron complexed heme acting as the enzymes catalytic center. **Figure 2B** depicts the monooxygenation reaction carried out by the CYP450 enzyme. The first step in the process is the binding of the substrate to the Type 1 binding site on the apoprotein coat. The binding of substrate then allows the sequential transfer of 2 electrons and oxygen (O₂) to the ferrous iron in the heme center. The source of the electrons comes from the transfer of two electrons from NADPH to the FAD/FMN containing cytochrome P450 reductase or to the cytochrome b5 reductase. The reductase then transfers one electron at a time to the CYP450 substrate complex. Subsequent removal of one molecule of oxygen creates a highly electronegative and highly reactive oxygen species bound to the high spin state of the ferric iron. The presence of this highly reactive oxygen in close proximity to the substrate ultimately leads to its incorporation and the monooxygenated substrate is released from the binding site.

CYP450 enzymes have a conserved heme functional group. The CYP450 isoforms differ based on the apoprotein coat, which allows for the binding of different substrates in a specific manner. There have been 18 gene families identified in mammals which encode for 57 individual CYP genes (Nebert and Russell 2002). There are only three families, *CYP1*, *CYP2*, and *CYP3*, which are responsible for the majority of CYP450-mediated drug metabolism. The work involved in our studies focuses on the CYP2E1 and CYP3A isoforms. The relative contributions of these isoforms to drug metabolism are presented in **Figure 3**, and the characteristics of these isoforms presented in **Table 2** (Bertz and Granneman 1997; Gonzalez 1997; Venkatakrishnan, Von Moltke et al. 2001; Rendic 2002; Lewis 2003).

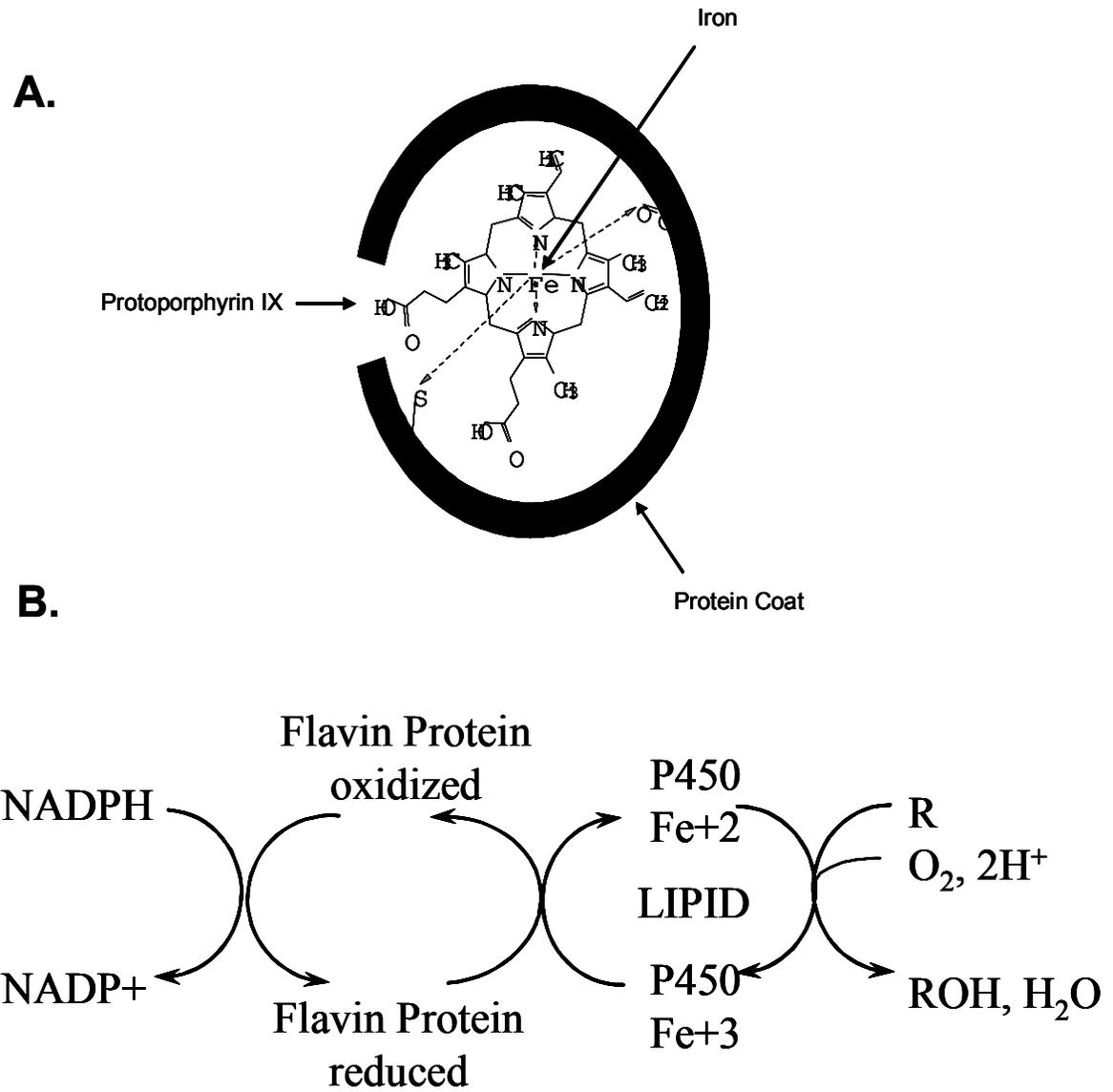


Figure 2: CYP450 structure and enzymatic reaction

Panel A is a schematic of a cytochrome P450 enzyme containing the apoprotein coat and iron containing heme catalytic center. Panel B depicts the enzymatic reaction carried out by CYP450 enzymes.

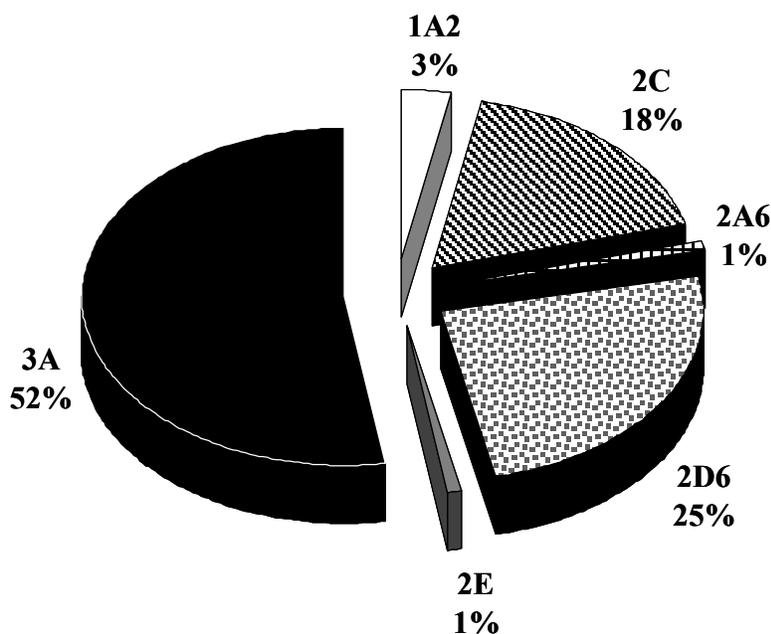


Figure 3: Contribution of the major human CYP450 isoforms to drug metabolism

Table 2: Major drug metabolizing CYP enzymes in humans

	Location	Substrates
CYP450		
1A2	Liver	Acetaminophen, caffeine, heterocyclic arylamines, theophylline
2A6	Liver	Coumarin
2B6	Liver, heart	Nicotine, bupropion
2C8	Liver, kidney	Tolbutamide, <i>R</i> -mephenytoin
2C9	Liver	Warfarin, phenytoin, tolbutamide, losartan, ibuprofen, flurbiprofen, indomethacin, diclofenac,
2C19	Liver, heart	Omeprazole, <i>S</i> -mephenytoin, diazepam
2D6	Liver, kidney	Codeine, debrisoquine, desipramine, dextromethorphan, fluoxetine, paroxetine, metoprolol, propranolol
2E1	Liver, kidney, brain	Ethanol, acetaminophen, chlorzoxazone, nitrosamines
3A4/5	Liver, intestine, kidney, brain, lung	Cortisol, testosterone, midazolam, cyclosporin, tacrolimus, cisapride, nifedipine, fluconazole, erythromycin, itraconazole, terfenadine, ketoconazole, verapamil, simvastatin

Abbreviations: CYP, cytochrome P450 isoform

Adapted from (Bertz and Granneman 1997; Gonzalez 1997; Venkatakrishnan, Von Moltke et al. 2001; Rendic 2002; Lewis 2003).

CYP2E1

CYP2E1 is the only isoform present in the CYP2E subfamily and contributes to 7% of total human hepatic CYP content. CYP2E1 metabolizes some important drugs including acetaminophen, ethanol, and chlorzoxazone, which are listed in **Table 2**. Interestingly, CYP2E1 plays a large role in endogenous substrate metabolism, including the metabolism of arachidonic acid (Adas, Salaun et al. 1999; Poloyac, Tortorici et al. 2004). Furthermore, CYP2E1 has the ability to metabolize xenobiotics to hepatotoxic or carcinogenic products (Lieber 1997). In rat, CYP2E1 demonstrates 78% amino acid homology with the human form of CYP2E1 (Rendic 2002). In both rats and humans the regulation of CYP2E1 is complex, involving transcriptional (via hepatocyte nuclear factor-1 α (HNF-1 α)), and post-translational (protein stabilization) events (Eliasson, Johansson et al. 1988; Eliasson, Mkrтчian et al. 1992; Roe, Poloyac et al. 2001). The CYP2E1 probe substrate, chlorzoxazone, has been shown to provide validated phenotypic analysis of both rat and human CYP2E1 activity both *in vivo* and *in vitro*, thereby allowing for comparison (Poloyac, Tosheva et al. 1999; Rockich and Blouin 1999; Tanaka 2001). Analysis of CYP2E1 in rats can also be correlated with humans and was one reason we chose to focus our attention on CYP2E1 in the work presented throughout this dissertation.

CYP3A

The *CYP3* family consists of four genes in humans, CYP3A4, CYP3A5, CYP3A7, and CYP3A43, (Venkatakrishnan, Von Moltke et al. 2001; Nebert and Russell 2002) and is considered the most important CYP family involved in the metabolism of drugs. CYP3A comprises 30 % of total liver CYP450 content and 70% of total intestinal CYP450 content, and metabolizes greater than 50% of all clinically relevant medications (Bertz and Granneman 1997;

Venkatakrishnan, Von Moltke et al. 2001). Human CYP3A4, which is the CYP3A isoform that carries out the metabolism of the majority of these substrates, is ~72% homologous with the rat CYP3A2 isoform and metabolizes similar substrates including testosterone and midazolam (Rendic 2002). The large inter-individual variability demonstrated with CYP3A4 activity may be due to differences in the expression pattern and metabolic capacity of CYP3A4 compared to other CYP3A isoforms as well as the existence of genetic polymorphism. CYP3A4 in the human is constitutively regulated via multiple transcriptional factors including CCAAT/enhancer binding protein (C/EBP α), and hepatic nuclear factor-3 (HNF3) (Rodriguez-Antona, Bort et al. 2003; Bombail, Taylor et al. 2004). Interestingly, CYP3A2 in the rat is regulated through transcriptional mechanisms via hepatic nuclear factor 4 (HNF-4) (Ogino, Nagata et al. 1999).

As depicted in **Figure 4**, alterations in CYP450 functional regulation due to genetic, environmental, drug co-administration, and/or disease state factors have all been shown to produce clinically significant effects on pharmacotherapeutic outcome. Each of these factors has been shown to alter the CYP450 enzyme system by increasing or inhibiting the enzymatic activity of individual or multiple isoforms. There has been extensive research performed investigating the effects of multiple disease states on CYP450 activity and expression (Morgan, Sewer et al. 1998; Blouin, Farrell et al. 1999). Interestingly, TBI, sepsis, and other models of inflammation have been shown to alter CYP450-mediated activity (Boucher and Hanes 1998; Poloyac, Perez et al. 2001; Roe, Poloyac et al. 2001; Harbrecht, Frye et al. 2005). These insults regulate CYP450 enzymes through the production of the acute phase response (APR), namely the release of cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α). (Toler, Young et al. 1993) The APR is a cascade of events which occurs following tissue damage due to traumatic or microbial insult. The APR results in local or

systemic effects, which affects nearly every organ through the APR mediators. These mediators include cytokines, complement, coagulation factors, antiproteases, transport proteins, growth factors, and other proteins (Gabay and Kushner 1999). Cytokines have been shown to dramatically alter the expression of CYP450s in multiple models. Of the pro-inflammatory cytokines, IL-6 mediates the majority of the hepatic alterations of the APR and has been shown to be the most potent inhibitor of CYP450s.

Intracellular signaling alterations following IL-6 receptor binding affect multiple transcription factors. The primary transcription factors activated during IL-6 binding are NF-IL6 (from the family of C/EBP binding proteins) and AP-1. Increases in IL-6 have been shown to produce decreases in the expression and activity of both CYP2E1 and CYP3A isoforms, through transcriptional alterations (Toler, Young et al. 1993). Many critical care insults, such as CA and TBI have been shown to produce increases in IL-6. In addition, IL-6 increases production of nitric oxide (NO), which has been shown to decrease the activity of multiple CYP450 isoforms (Carlson and Billings 1996; Khatsenko 1998; Binda, Lasserre-Bigot et al. 2003).

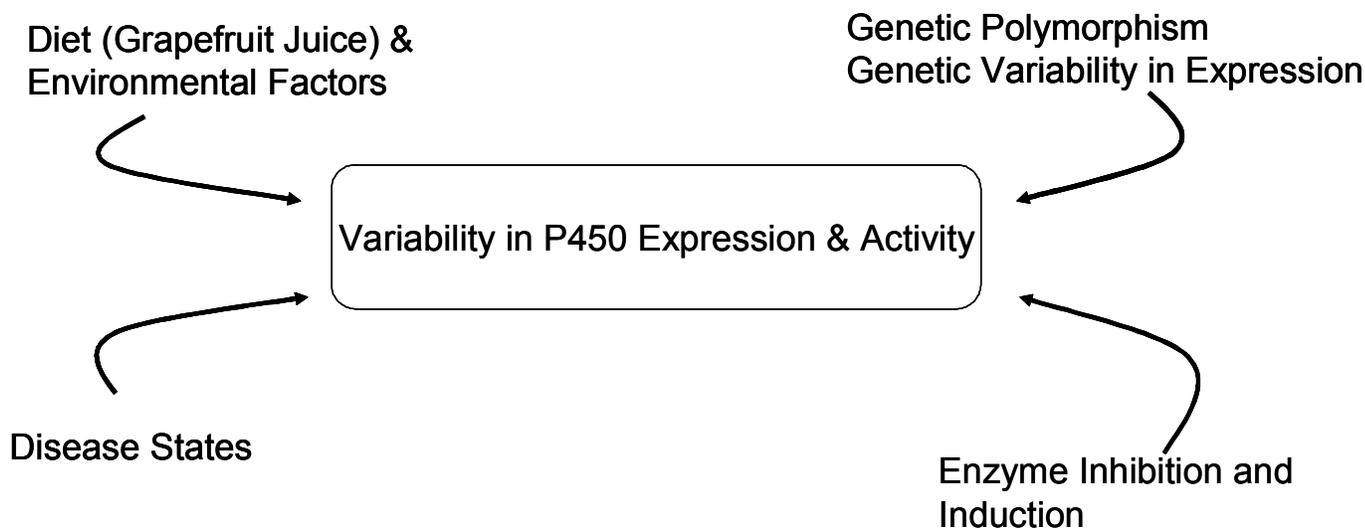


Figure 4: Variables affecting CYP450 expression and activity

1.4 THE EFFECTS OF THERAPEUTIC HYPOTHERMIA DURING COOLING ON DRUG DISPOSITION AND METABOLISM

Studies have evaluated both the effects of hypothermia during cooling, and the effects of hypothermia post-rewarming in the acute phase (immediately after re-warming, 5 hr after injury), sub-acute phase (12-24 hrs after injury), delayed phase (24-72 hrs after injury), and the chronic phase (>72 hrs after injury), on the disposition and metabolism of multiple medications. Hypothermia also alters drug response by affecting the interactions of the drug with its target receptor. In this chapter we will consider both the effects of hypothermia on drug metabolism during cooling and after re-warming, focusing on drugs germane to critical care medicine.

1.4.1 CYP450 Probe Substrates

Midazolam

Midazolam is a member of the benzodiazepine drug class and is used as an anesthetic adjunct and a hypnotic. It is exclusively metabolized by CYP3A4 and CYP3A5 and is used as a probe substrate to assess CYP3A4 activity in human subjects and for *in vitro* analysis (Yuan, Madani et al. 2002; Eap, Bouchoux et al. 2004). Fukuoka *et al.* demonstrated that as TBI subjects core temperature was lowered below 35°C, midazolam plasma concentrations were ~5-fold higher compared to when core body temperature was above 35°C.(Fukuoka, Aibiki et al. 2004) This study was conducted in 15 TBI Japanese subjects, with an average GCS score of 7.3 ± 1. Eight of these subjects were treated with mild hypothermia (32°C-34°C) and seven subjects were maintained at normothermic temperatures (37°C). Each subject was started on a continuous infusion of midazolam at 5µg/kg/min. Subjects who were maintained normothermic temperatures achieved steady state concentrations of ~1500 ng/ml. Interestingly, the

hypothermia group demonstrated a dramatic increase in midazolam concentrations when body temperature was below 35°C. Pharmacokinetic analysis demonstrated ~100-fold decrease in Cl_S of midazolam in the period when subjects' core temperature was less than 35°C. The well-stirred model of hepatic clearance (Cl_H) states that for probe substrates, which are typically low clearance drugs and exclusively metabolized by CYP-mediated mechanisms, the Cl_H approximates the Cl_S which is dependent upon the fraction of unbound drug and the intrinsic clearance of the CYP450 isoform (Pang and Rowland 1977; Pang and Rowland 1977; Pang and Rowland 1977). Therefore, since midazolam is a low clearance drug and exclusively metabolized by CYP3A4 and CYP3A5, higher midazolam concentrations are likely due to either depressed CYP3A4 activity or a decrease in the protein binding when a subject's core temperature is mildly hypothermic.

1.4.2 Drugs Commonly Used in the ICU

Anesthetics/Sedatives

Fentanyl

Fentanyl is clinically used as an analgesic and is a member of the opioid drug class. Like midazolam, fentanyl is primarily metabolized by CYP3A4; however it is classified as a high clearance drug with a large volume of distribution. According to the well-stirred model, Cl_H of high clearance drugs, is primarily dependent upon hepatic blood flow (Pang and Rowland 1977; Pang and Rowland 1977; Pang and Rowland 1977). Since fentanyl is commonly used clinically, there has been considerable research on the effects of hypothermia on fentanyl concentrations in both experimental models as well as patients (Lunn, Stanley et al. 1979; Bovill and Sebel 1980;

Koska, Romagnoli et al. 1981; Koren, Barker et al. 1987; Gruber, Laussen et al. 2001; Pettifer and Hosgood 2004; Kussman, Zurakowski et al. 2005).

With the onset of CPB, fentanyl concentrations have been shown to be lowered by 53% (Bovill and Sebel 1980). Other studies have also demonstrated dramatic alterations in the plasma concentrations of fentanyl during CPB (Lunn, Stanley et al. 1979; Koska, Romagnoli et al. 1981). Lunn *et al.* was one of the first to measure plasma fentanyl concentrations during CPB (Lunn, Stanley et al. 1979). Eighteen patients undergoing two-vessel coronary artery bypass were cooled to 32°C during extracorporeal support and re-warmed to 37°C at its conclusion. Fentanyl (75µg/kg IV) was administered and plasma concentrations were 37% lower at the onset of CPB compared to pre-CPB concentrations. As patients were made hypothermic during CPB surgery, fentanyl concentrations remained unchanged and gradually increased over time. This study did not perform a pharmacokinetic analysis on this data. The initial low fentanyl plasma concentrations could be due to hemodilution via the administration of large volumes of fluids commonly performed during CPB. Secondly, once hypothermia was started during CPB, concentrations were higher, which could be due to either a decrease in hepatic blood flow during surgery or a decrease in hepatic metabolic activity, both of which were not measured.

A study by Koska *et al.* demonstrated that fentanyl concentrations were higher during CPB surgery (Koska, Romagnoli et al. 1981). This study investigated the pharmacokinetics of low dose (5-8 µg/kg) fentanyl in two groups of six patients, one group undergoing CPB surgery and one group not undergoing CPB surgery (Koska, Romagnoli et al. 1981). These authors did not report body temperature, however they noted three important findings; 1) a rise of ~30% in fentanyl concentrations after CPB surgery termination, 2) a prolonged elimination half-life of fentanyl after CPB, 5.2 ± 2.7 hours compared to 3.3 ± 1.1 hours in surgical controls, and 3) a

decrease of 30% in hepatic perfusion as measured by indocyanine green, both during and after CPB (Koska, Romagnoli et al. 1981). The authors concluded that in this study, the higher plasma concentrations and the prolongation of the $t_{1/2}$ are due to decreases in hepatic blood flow and as well as hepatic metabolic function.

In most animal studies of therapeutic hypothermia in experimental TBI demonstrating neuronal protection, isoflurane anesthesia is used as the anesthetic (Kochanek and Safar 2003). However, Statler *et al* demonstrated that hypothermia was not neuroprotective after experimental TBI in rats anesthetized with fentanyl. Surprisingly, an increase in lesion volume vs. normothermia was observed under fentanyl anesthesia (Statler, Alexander et al. 2003). In addition serum concentrations of fentanyl were higher in the hypothermic vs. the normothermic animals. High fentanyl concentrations have been shown to produce electrical seizure activity and neurological damage in patients (Tempelhoff, Modica et al. 1992). One possible explanation of these results is that higher fentanyl concentrations in the brain may have increased damage in experimental brain injury (Kofke, Garman et al. 1999). This study demonstrates the need for full knowledge of the potential pharmacological effects of therapeutic hypothermia in order to optimize its use both in clinical use and in further therapeutic trials.

There have been a number of studies which have investigated the effects of therapeutic hypothermia on fentanyl concentrations in pediatric patients undergoing cardiac surgery. Koren *et al.* (Koren, Barker et al. 1987) demonstrated fentanyl concentrations to be unchanged in 18 pediatric patients undergoing cardiac surgery who were made hypothermic (18°C – 25°C), compared to pre-surgery concentrations. Patients received a fentanyl IV bolus of 30-50 µg/kg followed by a continuous IV infusion of 0.15 – 0.30 µg/kg/min and profound hypothermia (<20°C) was induced at the commencement of CPB. This study discontinued the infusion of

fentanyl at the initiation of CPB and hypothermia, in order to evaluate the effect of hypothermia without confounding influences of the infusion. Since the infusion was stopped and there were no changes in plasma fentanyl concentrations observed during this period, these data demonstrated that during profound hypothermia, the Cl_S of fentanyl was very low. Even if hepatic blood flow was decreased by hypothermia and CPB, there still should have been metabolism occurring resulting in a decrease of fentanyl concentrations, unless profound hypothermia produced complete inhibition of CYP3A4 enzymatic activity.

Koren *et al.* performed a study in piglets to identify the effects of hypothermia on fentanyl concentrations, without the multiple variables of CPB surgery. Results showed that fentanyl plasma concentrations were considerably higher in hypothermic (29°C) versus normothermic (37°C) piglets. Furthermore, fentanyl Cl_S was decreased during hypothermia (0.65 ± 0.12 ml/kg/min) compared to normothermia (2.42 ± 0.22 ml/kg/min). The authors concluded that the decrease in fentanyl Cl_S in piglets could be due to decreases in cardiac output resulting in a decrease in liver blood flow, as the cardiac output has been shown to be decreased by 42% during hypothermia (29°C) (Koren, Goresky *et al.* 1984). However the decrease in fentanyl Cl_S in this study (73%) due to hypothermia far exceeded the decrease in cardiac output previously reported (42%). It is likely that hypothermia decreases CYP3A-mediated metabolism of fentanyl in addition to the observed blood flow alterations.

Kussman *et al.* (Kussman, Zurakowski *et al.* 2005) showed that plasma fentanyl concentrations were significantly lower at the onset of CPB and then recovered to pre-CPB levels after 30 minutes in infants. This study measured changes in fentanyl concentrations during infant cardiac surgery using a bypass circuit and examined the relationship of plasma fentanyl concentrations and temperature to Bispectral Index (BIS) as index of conscious level

(Kussman, Zurakowski et al. 2005). Fifteen neonates and infants undergoing cardiac surgery with hypothermic CPB were administered a bolus dose of fentanyl, 30µg/kg, followed by a continuous infusion of 0.3 µg/kg/min. Hypothermia was defined as mild (>32°C), moderate (25°C – 32°C), or deep (<25°C). Median plasma fentanyl concentrations were significant lowered from 17 ng/ml, at aortic cannulation, to 10 ng/mL after 4 minutes of CPB. However, after 30 minutes of CPB, plasma fentanyl concentrations were significantly higher and remained constant for the remainder of the surgery. Results also showed that there was no correlation of BIS to fentanyl concentrations and temperature and BIS continued to decrease throughout the entire study period. There was no pharmacokinetic or hepatic blood flow analysis performed in this study to identify the mechanisms behind these alterations; however these results are consistent with the previous mentioned studies (Lunn, Stanley et al. 1979; Koska, Romagnoli et al. 1981; Koren, Barker et al. 1987).

Another study by Gruber *et al.*, demonstrated that fentanyl concentrations were higher during the hypothermic period in infants undergoing corrective cardiac surgery (Gruber, Laussen et al. 2001). Forty-five neonates and infants, less than 6 months of age, undergoing deep hypothermic cardiopulmonary bypass (DHCB) were included in this prospective, randomized double-blinded study. Patients were randomized to one of three groups, Group 1 received fentanyl (25µg/kg) IV bolus (n=15), on initiation of DHCB. Group 2 received fentanyl IV infusion (bolus dose:25µg/kg; infusion rate:10µg/kg/hr) upon initiation of CPB. Group 3, n=15, received IV bolus of fentanyl (25µg/kg) and IV bolus of midazolam (200µg/kg) followed by midazolam IV infusion (100 µg/kg/hr) and fentanyl IV infusion (10 µg/kg/hr), upon initiation of DHCB. The results showed that when subjects in both Groups 2 and 3, were made hypothermic during DHCB, the fentanyl concentrations were significantly higher at the end of surgery.

Furthermore, there were multiple physiological changes during DHCB including increases in epinephrine, norepinephrine, and cortisol plasma concentrations, which the authors conclude is due to an increase in the stress response during surgery. However, the change in cortisol is in contrast to previous studies, which actually have shown a decrease in cortisol concentrations with high dose opioid administration during cardiac surgery (Anand and Hickey 1992). Another explanation is a decrease in the metabolism of cortisol to 6 β -hydroxycortisol by the CYP3A4 isoform (Ged, Rouillon et al. 1989; Baron, Goh et al. 2001). The ratio between cortisol and 6 β -hydroxycortisol, has been used as a measure of CYP3A4 activity (Joellenbeck, Qian et al. 1992). Despite the fact that 6 β -hydroxycortisol was not measured in this study and that only 1% of all cortisol metabolites recovered in the urine are the 6 β -hydroxy metabolite, these changes in cortisol levels could potentially be due decreases in CYP3A4 activity.

Collectively, these studies demonstrate two important findings. First, there appears to be consistent findings of the effects of CPB on fentanyl concentrations in both infants and adults. The above mentioned studies demonstrated an initial decrease in fentanyl concentrations at the initiation of CPB, due to hemodilution, followed by an increase, likely due to the hypothermic period during CPB. However, the complexity of CPB surgery makes it difficult to clearly identify the mechanisms behind the observed alterations, as there are multiple factors which can influence plasma fentanyl concentrations including; the priming volume of the circuit, binding of fentanyl to the circuit tubing and membrane oxygenator, sequestration of fentanyl within pulmonary circulation, altered protein binding after hemodilution, and variable metabolism and excretion secondary to hypothermia (Kussman, Zurakowski et al. 2005). Therefore, in the CPB model, it is difficult to quantify the contribution of these variables to alterations in fentanyl concentrations. However, inhibition of fentanyl metabolism was also examined in a carefully

controlled study in piglets to identify the effects of hypothermia on fentanyl concentrations, without the multiple variables of CPB surgery (Koren, Barker et al. 1987). Fentanyl plasma concentrations were ~2-fold higher in hypothermic (29°C) vs. normothermic piglets (37°C). Furthermore, the Cl_S of fentanyl was decreased ~3.7-fold during hypothermia compared to normothermia. The decrease in Cl_S in piglets was caused by a decrease in cardiac output with reduced hepatic perfusion resulting in a decrease in hepatic blood flow (Koren, Goresky et al. 1984). However, the decrease in Cl_S in this study (73%) far exceeded the decrease in cardiac output previously reported (42%). It is likely that hypothermia-produced decreases the CYP3A-mediated metabolism of fentanyl in addition to the observed blood flow effects.

Remifentanyl

Remifentanyl is a derivative of fentanyl and is a potent opioid and an anesthetic adjunct with a short half-life due to rapid hydrolysis to a minimally active metabolite by nonspecific blood and tissue esterases (Burkle, Dunbar et al. 1996; Hoke, Cunningham et al. 1997). Michelsen *et al.* reported that the Cl_S of remifentanyl decreased as temperature was decreased below 37°C in 68 patients undergoing CPB with hypothermia (Michelsen, Holford et al. 2001). Remifentanyl was administered at 1, 2, or 3 $\mu\text{g}/\text{kg}/\text{min}$ and CPB was conducted with varying degrees of hypothermia. Remifentanyl Cl_S decreased by 6.37% for each degree Celsius below 37°C. The decrease in Cl_S was likely due to a hypothermia-mediated decrease in remifentanyl metabolism by blood and tissue esterases. In addition, this study simulated remifentanyl concentrations for two hypothetical 70-kg patients receiving an infusion of 1 $\mu\text{g}/\text{kg}/\text{min}$ for 60 minutes at which time CPB was initiated with cooling and maintenance of temperature at 32°C or 27°C. The simulation demonstrated that there was a proportional decrease of 6.37% in Cl_S

with each degree Celcius in temperature below 37°C, with a prediction error for all data points of 17.5%. Both simulations demonstrated that under moderate hypothermia, the infusion rate of remifentanil can be decreased almost immediately after the institution of CPB to achieve target blood levels. For mild hypothermia, the infusion rate may be decreased after ~20-30 min. Collectively, target remifentanil concentrations may be obtained by decreasing the infusion rate approximately 30% for each 5°C decrease in temperature. This study was one of the first to not only demonstrate hypothermia-induced increases in remifentanil concentrations but to provide a potential dosing nomogram for remifentanil for clinicians use. Currently, no such dosing algorithms exist for any drug for the use of mild hypothermia after CA and TBI.

Pentobarbital & Phenobarbital

Pentobarbital is a member of the barbiturate drug class and is metabolized primarily via side-chain oxidation by multiple CYP450 isoforms. One of the first studies to investigate the effects of hypothermia on drug metabolism demonstrated that in an isolated perfused rat liver maintained at 30°C, 25°C, or 20°C, the metabolism of pentobarbital was decreased by half, one-third, and one-fourth the rate, respectively, vs. normothermia (Kalser, Kelly et al. 1969). In the same study, radioactive C¹⁴-pentobarbital was infused at a dose of 0.5 to 1 mg into an isolated perfused rat livers maintained at 37°C, 30°C, 25°C, or 20°C. The rate of uptake of pentobarbital in the liver was also decreased in hypothermic compared to normothermic preparations. The rate of metabolism was inversely proportional to temperature and hypothermia markedly reduced the amount of pentobarbital metabolites of appearing in blood and the bile.

Kadar *et al* performed a study in critically injured children and found that moderate hypothermia decreases the metabolism of phenobarbital (Kadar, Tang et al. 1982). Four boys

between the ages of 5 and 15 years suffering from TBI were treated with moderate hypothermia (30°C to 31°C) for six to nine days. Urinary concentrations of phenobarbital and its metabolites were determined during the hypothermic period and during the normothermic period. There was a 48% decrease in the rate of urinary excretion of hydroxyphenobarbital during hypothermia versus normothermia. The urinary excretion rate of the parent drug was higher (~52%) during hypothermia vs. normothermia. Since the urinary excretion rate of hydroxyphenobarbital was decreased and phenobarbital was increased when the core temperature was lowered, this lends evidence that hypothermia likely decreased the rate of metabolism of phenobarbital.

Propofol

Propofol is used as a sedative in adult critical care and is primarily metabolized by the CYP2B6 isoform and further glucuronidated by UDP-glucuronosyltransferase (UGT) 1A9. Hypothermia increases propofol concentrations in normal healthy volunteers and patients on CPB (Russell, Wright et al. 1989; Leslie, Sessler et al. 1995; Leslie, Bjorksten et al. 2002). A study in 1989 set out to investigate the effects of CPB on propofol concentrations (Russell, Wright et al. 1989). Ten males were scheduled for coronary artery surgery. Propofol was administered as continuous infusion at a rate of 10 mg/kg/hour and then reduced to 3 mg/kg/hour for approximately 250 minutes. Upon initiation of CPB, propofol concentrations decreased sharply as a result of hemodilution, which has been previously shown with other medications (Lunn, Stanley et al. 1979; Koska, Romagnoli et al. 1981). During the period of deep hypothermia (25°C-27°C), which lasted approximately 50 minutes, propofol concentrations were consistently higher. The reason for these changes could be due to a decrease in propofol metabolism via CYP450-mediated mechanisms, as well as a decrease in hepatic blood flow.

Leslie *et al.* performed a series of studies investigating the effects of mild hypothermia on propofol blood concentrations (Leslie, Sessler et al. 1995; Leslie, Bjorksten et al. 2002). The first study demonstrated that propofol concentrations were dramatically higher when hypothermia was induced in normal healthy volunteers (Leslie, Sessler et al. 1995) Six healthy volunteers were administered an IV bolus of 1 mg/kg followed by a 4-hour infusion at 0.5 mg/kg/hr and core temperature was maintained at either 37°C or cooled to 34°C. Indocyanine green was administered as a 1 mg IV bolus followed by a 12 mg/hr infusion for hepatic blood flow assessment. Results demonstrated that propofol concentrations were 28% higher at 34°C compared to 37°C. Using a three-compartment model, total body clearance, central and compartmental V_d were not changed, however inter-compartmental clearance was significantly decreased during hypothermia. Thus, mild hypothermia increased propofol concentrations by decreasing propofol metabolism, either P450 or UGT1A9-mediated, without altering hepatic blood flow or V_d . While no differences in the hepatic blood flow were observed, these results suggest that the higher propofol concentrations observed during hypothermia were due to a decrease in propofol metabolism. The follow-up study investigated the effects of mild hypothermia on propofol requirements in patients undergoing intracranial tumor surgery (Leslie, Bjorksten et al. 2002). Forty patients were anesthetized with a propofol infusion and an IV bolus of alfentanil, and were randomized to a core temperature 37°C or 34°C. The results demonstrated that in the hypothermia group, propofol concentrations were significantly lower than compared to the normothermia group, 2.25 (1.5-3.5) µg/ml versus 3.25 (2.0-4.5) µg/ml, respectively. Furthermore, propofol concentrations, but not core temperature, predicted loss of response to command (odds ratio, 11.76). Core temperature did not alter the relationship between BIS and response to command. The authors concluded that propofol infusion regimens

may not require adjustment during mild hypothermia for patients undergoing intracranial tumor surgery.

These two studies report contrasting results regarding the effects of mild hypothermia on propofol pharmacokinetics and pharmacodynamics. Further investigation of the differences in these studies may explain the variable results. The first study set out to examine the effects of mild hypothermia on the pharmacokinetics of propofol in healthy volunteers. They demonstrated that core temperature was associated with higher propofol concentrations. The second study set out to examine the effects of mild hypothermia on propofol requirement measured as response to commands in neurosurgical patients. In this study, the authors targeted different concentrations in the hypothermia group versus the normothermia group (2.25 µg/mL versus 3.25 µg/mL, respectively). Therefore, the dose of propofol was dictated by the response of the patient; however the doses of propofol administered were not indicated. Therefore, we can conclude from these studies that 1) mild hypothermia increased propofol concentrations by decreasing propofol metabolism without altering hepatic blood flow and 2) the target propofol concentrations were less in patients treated with mild hypothermia compared to patients treated under normothermic conditions to achieve the same response, potentially due to alterations in the pharmacodynamic response of propofol, which will be discussed later in this chapter. Clinically, we can conclude that lower target plasma concentrations are needed to provide a similar response.

Isoflurane

Isoflurane is a member of the volatile anesthetic drug class. Previous studies have demonstrated that hypothermia decreases the minimal alveolar concentrations (MAC) of volatile

anesthetics such as halothane and isoflurane required for clinical response potentially due to alterations in the metabolism resulting in higher concentrations present (Vitez, White et al. 1974). Extrapolation of these results indicates that hypothermia itself would act as a complete anesthetic at temperature between 18-21°C (Regan and Eger 1967). Antognini *et al.* determined that when temperature is decreased, the anesthetic requirements of isoflurane are decreased (Antognini 1993). Eight female goats were anesthetized with isoflurane and underwent CPB surgery. MAC was initially determined at baseline and re-determined when animals were cooled to 29°C. At this time animals were then further cooled to 20°C, at which time MAC was re-assessed. The results showed that as temperature was lowered, the MAC was also decreased. Furthermore, when the temperature reached 20.1°C, anesthetic requirements of isoflurane were completely eliminated. The authors referred to the fact that these differences in MAC could be due to temperature-induced changes of the solubility of isoflurane in lipid materials. However, they stated that this conclusion is speculative and there may be other mechanisms behind these alterations, such as a decrease in metabolism. Expanding upon this concept, it is known that isoflurane is metabolized by CYP2E1 (Kharasch, Hankins et al. 1999). The work described in Chapter 2, demonstrates that moderate hypothermia (30°C) decreased the Cl_s of a CYP2E1 probe substrate, chlorzoxazone, in a CA rat model by ~2-fold (Tortorici, Kochanek et al. 2006). Furthermore, we found that the mechanism behind this alteration in the Cl_s of chlorzoxazone was a decrease in the intrinsic clearance (Cl_{int}) of CYP2E1. A decrease in the CYP2E1-mediated metabolism of isoflurane will likely contribute to the decrease in MAC due to hypothermia.

Neuromuscular Blockers

Vecuronium

Neuromuscular blocking agents are commonly used in the ICU. During the use of mild hypothermia they are often employed to prevent shivering. Vecuronium is primarily eliminated via the liver by carrier-mediated transport (p-glycoprotein) and metabolized by CYP450 enzymes (Smit, Schinkel et al. 1998). Studies have investigated the effects of hypothermia on vecuronium pharmacokinetics and pharmacodynamics in patients undergoing elective surgery (Heier, Caldwell et al. 1991; Heier, Caldwell et al. 1994). In these studies, surgical patients treated with hypothermia demonstrated a 3-fold increase in the duration of action of vecuronium as compared to normothermia patients (Heier, Caldwell et al. 1991; Heier, Caldwell et al. 1994).

Caldwell *et al.*, also evaluated the effects of hypothermia on vecuronium pharmacokinetics (Caldwell, Heier et al. 2000). Healthy volunteers were randomized to $<35^{\circ}\text{C}$, $35.0\text{-}35.9^{\circ}\text{C}$, $36.0\text{-}36.9^{\circ}\text{C}$, and $\geq 37^{\circ}\text{C}$. Hypothermia reduced the plasma clearance of vecuronium by 11.3% per $^{\circ}\text{C}$. Hypothermia also decreased the rate constant for equilibration between plasma and effect site (0.023 min^{-1} per $^{\circ}\text{C}$). Furthermore, the slope of the concentration-response curve was increased during hypothermia by 0.43 per $^{\circ}\text{C}$, indicating that recovery of neuromuscular function will be delayed by hypothermia. However, tissue sensitivity was not influenced by core temperature. Reduced clearance can explain the increased duration of action vecuronium with reducing core temperature that was previously demonstrated in the study conducted by Heir *et al.* (Heier, Caldwell et al. 1991). Furthermore, the authors concluded that the pharmacokinetic alterations observed could be due to changes in hepatic metabolism.

Rocuronium

Rocuronium is primarily eliminated via the bile. Beaufort *et al.* examined the influence of hypothermia on the time-course of action and the pharmacokinetics of rocuronium in human

neurosurgical patients (Beaufort, Wierda et al. 1995). The duration of action of rocuronium was increased by 5 min per °C as measured by neuromuscular function. Pharmacokinetic analysis revealed a ~2-fold decrease in the Cl_s of rocuronium with hypothermia. Hypothermia significantly prolonged the time-course of action of rocuronium and influenced the pharmacokinetics of rocuronium by decreasing the Cl_s . Hypothermia also decreases the biliary excretion of other drugs, such as atropine, thereby, suggesting that biliary transport mechanisms of elimination may be similarly reduced during hypothermia in patient (Kalser, Kelvington et al. 1965).

Atracurium

Atracurium is metabolized by non-enzymatic decomposition in the blood or by Hoffman elimination. An alternative elimination pathway is via enzymatic ester hydrolysis (Fisher, Canfell et al. 1986). Leslie *et al.* observed that mild hypothermia (34°C) significantly increased the duration of action of atracurium in normal healthy volunteers (Leslie, Sessler et al. 1995). Six volunteers (2 women and 4 men) were studied on two randomly assigned days, one where core temperature was decreased to 34°C and one where core temperature was maintained at 37°C. Atracurium was administered as an IV bolus of 0.5 mg/kg and hypothermia was induced via surface cooling for approximately 200 minutes. Hypothermia significantly prolonged the time to recovery of the first twitch in the train-of-four test from 44 ± 4 minutes to 68 ± 7 minutes.

Despite the fact that the three above mentioned neuromuscular blocking agents are eliminated by different pathways, each agent demonstrated an increase in duration of action by mild hypothermia caused by decreases in drug Cl_s . In light of the significant increase in the

duration of action, these studies indicate that clinicians using neuromuscular blocking agents in hypothermic patients should reduce doses and monitor neuromuscular function to avoid overdose.

Cardiovascular Drugs

Atropine

Atropine, a competitive antagonist of the actions of acetylcholine at the muscarinic receptors, is commonly used for intubation and is also used in cardiopulmonary resuscitation. Atropine is eliminated primarily via the kidneys with some contribution by biliary excretion. There were a series of studies in the 1960s which were the first to investigate the effects of hypothermia on hepatic drug metabolism, biliary excretion, and hepatic uptake of drugs (Kalser, Kelvington et al. 1965; Kalser, Kelvington et al. 1965; Kalser, Kelvington et al. 1968; Kalser, Kelvington et al. 1968; Kalser, Kelly et al. 1969). The first of these studies showed that moderate hypothermia decreased biliary excretion of atropine in the nephrectomized rat.(Kalser, Kelvington et al. 1965) A follow-up study in 1965, determined that hypothermia decreases the uptake, metabolism, and excretion of atropine in the isolated perfused rat liver (Kalser, Kelvington et al. 1965). Lastly, it was shown that as temperature was decreased to 25°C and 17°C, there was an increase in the percent of atropine recovered in the liver, indicating decreased transport out of the liver. These results could be due to a decrease in hepatic blood and/or bile flow as temperature is decreased. Therefore, hypothermia decreases four processes in drug disposition and excretion of atropine; blood flow, uptake of drug, metabolism of drug, and excretion of atropine.

Propranolol

Propranolol is a β -adrenoreceptor antagonist that is commonly used in the ICU (Carmona, Malbouisson et al. 2005). Propranolol is predominately metabolized by CYP2D6. Hypothermia alters plasma propranolol levels in models of CPB (McAllister, Bourne et al. 1979; McAllister and Tan 1980; Carmona, Malbouisson et al. 2005). Carmona *et al.* demonstrated that mild hypothermia increased the $t_{1/2}$ and V_d of propranolol in CPB patients (Carmona, Malbouisson et al. 2005). Eleven patients receiving propranolol before surgery (80-240 mg) and postoperatively (10mg) were evaluated. Mild hypothermia (32-34°C) was started after initiation of CPB and maintained until the end of coronary grafting. Results demonstrated that there was an increase in $t_{1/2}$ and V_d , post-operatively compared to pre-operatively, and no changes were observed in the Cl_S at these time-points. The authors concluded that the increase in $t_{1/2}$ is possibly due to mild hypothermia effects on hepatic metabolism of propranolol. Furthermore, the changes in the V_d could be due to the hemodilution effects of CPB, making it difficult to identify the effects of hypothermia on drug metabolism compared to the direct effects of CPB. (Kussman, Zurakowski et al. 2005).

McAllister *et al.* demonstrated that deep hypothermia in patients undergoing CPB resulted in higher propranolol concentrations compared to patients who were normothermic (McAllister, Bourne et al. 1979). However, the variability due to CPB surgery did not allow them to perform a formal pharmacokinetic analysis. Therefore, they extended the study to evaluate propranolol kinetics in mongrel dogs undergoing CPB surgery at deep hypothermia (26°C) after IV bolus administration. Dogs maintained under deep hypothermic conditions demonstrated a marked decrease in the Cl_S of propranolol compared to paired normothermic controls, 0.0323 ± 0.0072 L/kg/min vs. 0.0644 ± 0.0110 L/kg/min, respectively. Furthermore,

the V_d and $t_{1/2}$ were decreased and the elimination rate constant of propranolol was significantly increased.

In order to evaluate the mechanisms behind these alterations in Cl_s of propranolol, McAllister *et al.* performed an enzyme kinetic study in rat liver microsomes incubated with propranolol, at 37°C and 26°C, which match the temperatures used in their animal study (McAllister and Tan 1980). They demonstrated that when rat liver microsomes were incubated with propranolol at hypothermic temperatures (26°C), there was a decrease in metabolism at all concentrations compared to normothermia (37°C) (McAllister and Tan 1980). Furthermore, the mechanism behind this decrease in metabolism was due to alterations in the affinity of both substrates (propranolol and verapamil) for the CYP2D6. There were no changes in the V_{max} for both substrates. The work presented in chapter 2 demonstrates similar results in CYP2E1-mediated chlorzoxazone (CZN) metabolism in rat liver microsomes incubated at normothermia (37°C) or moderate hypothermia (30°C) (Tortorici, Kochanek et al. 2006). We showed a 2-fold increase in the K_m and no change in V_{max} when rat liver microsomes were incubated at moderate hypothermic temperatures (Tortorici, Kochanek et al. 2006). This series of studies provides evidence that the application of hypothermia decreases the Cl_s of propranolol and the mechanism of this decrease is due to changes in the activity of CYP2D6. Furthermore, the decrease in the activity of CYP2D6 is due to a decrease in the affinity of CYP2D6 for the substrate, propranolol.

Anticonvulsants

Phenytoin

Phenytoin is a commonly used anticonvulsant in the ICU. A unique characteristic of the disposition of phenytoin is the known saturable hepatic metabolism observed within the typical therapeutic range. The result of this saturable metabolism is non-linear increases in phenytoin

plasma concentrations with increases in dose. The Cl_s of phenytoin depends primarily on the unbound drug fraction and the activity of hepatic CYP2C9 and CYP2C19. The metabolism of phenytoin is influenced by many factors, such as the administration of other drugs, changes in albumin levels, and concurrent illnesses. Iida *et al.* examined the effects of hypothermia on the pharmacokinetics of phenytoin in patients with TBI (Iida, Nishi et al. 2001). Fourteen patients were cooled to 34°C and phenytoin was given as a loading bolus dose. Patients were also dosed with phenytoin 7-8 days after injury when core temperature was normothermic (36°C). Both total and unbound phenytoin concentrations were higher at 34°C than at 36°C, consistent with a reduction in the intrinsic clearance of the drug by liver enzymes. In addition, the AUC was increased by 180% at 34°C versus 36°C. The elimination rate constant, k_e , and the Cl_s were decreased by 50% and 67% at 34°C compared to 36°C, respectively. Furthermore, the plasma concentrations of phenytoin metabolites were markedly lower at 34°C compared to 36°C. No change was observed in the plasma protein binding of phenytoin. This study demonstrated three important findings; first, hypothermia dramatically altered the pharmacokinetics of phenytoin. Second, the decrease in Cl_s was due to decreased CYP450 metabolism and was not due to alterations in plasma protein binding. Third, the concentration of the metabolites was reduced in hypothermia vs. normothermia, providing further evidence that a decrease in CYP450 activity was an important mediator of the observed pharmacokinetic changes. In light of the common use of phenytoin in neurointensive care, vigilance towards monitoring for toxic levels of this agent should be heightened during use of hypothermia.

Theophylline

One study that did not demonstrate a significant change in CYP450-metabolized drug elimination was a study conducted by Koren *et al.* investigating moderate hypothermia effects on

theophylline concentrations in piglets (Koren, Barker et al. 1985). Theophylline is a bronchodilator and is a drug with a narrow therapeutic index and an increased chance for toxicity and adverse events, such as tachycardia, cardiac arrhythmias, palpitations, seizures, agitation, depression and nervousness. The primary mechanism of elimination of theophylline is via hepatic metabolism through the CYP1A2 and CYP3A4 isoforms and to a smaller extent, the CYP2E1 isoform. In seven piglets treated with moderate hypothermia (29°C), theophylline was administered as an IV bolus. Results demonstrated that there were no changes in the Cl_s and elimination rate constant in hypothermic animals compared to normothermic animals. This study also demonstrated that cardiac output was decreased in a linear fashion as temperature was also decreased. Since theophylline is a low extraction drug and there is no change in the pharmacokinetics of theophylline in this animal model, the authors concluded that moderate hypothermia does not alter CYP450 metabolism of theophylline. However, the results of this study were limited by the short duration of plasma sampling and the timing of sampling relative to induction of hypothermia. For example, the duration of blood collection in both normothermic and hypothermia piglets was 360 minutes. However, the calculated $t_{1/2}$ in normothermic animals was 1019 ± 285 minutes, which is ~ 2.5 times greater than the actual sampling time. This short sampling time results in the estimates of AUC only during the initial period after dosing without the ability to determine the influence of hypothermia on hepatic clearance. A longer duration of sampling in future studies under continuous infusion dosing regimens would allow for an appropriate determination of the effects of moderate hypothermia on theophylline elimination.

Antimicrobials

There have been several studies which have investigated the effects of hypothermia on the renal clearance (Cl_R) of antimicrobial agents in multiple models (Koren, Barker et al. 1985; Klamerus, Rodvold et al. 1988). Koren *et al.* performed a study investigating the effect of hypothermia on the pharmacokinetics of gentamicin in piglets (Koren, Barker et al. 1985). Gentamicin is an aminoglycoside antimicrobial agent that is almost completely renally excreted unchanged. In seven piglets treated with moderate hypothermia (29°C), gentamicin was administered as an IV bolus dose of 5 mg/kg. The serum concentrations of gentamicin were consistently higher in hypothermic piglets compared to normthermic piglets. The $t_{1/2}$ of gentamicin was increased from 135 ± 19 min in piglets kept normothermic to 187 ± 7 min in piglets were treated with moderate hypothermia. Furthermore, this study showed a rank order decrease in cardiac output as temperature is decreased, which could cause a decrease in the glomerular filtration rate. Since gentamicin is both filtered and secreted, a decrease in the GFR contributes to an alteration in the Cl_R of gentamicin. The authors suggested that the decrease in cardiac output due to hypothermia could be a potential mechanism of a decrease elimination of gentamicin in hypothermic piglets.

Klamerus *et al.* performed a study in 10 patients undergoing elective coronary artery bypass grafting or cardiac valve replacement surgery investigating the effects of CPB on the disposition of vancomycin and netilmicin (Klamerus, Rodvold et al. 1988). Vancomycin is a glycopeptide antimicrobial agent commonly used in clinical practice and is eliminated mainly through renal excretion. Netilmicin is a member of the aminoglycoside antibiotic class and is also primarily elimination through renal excretion. CPB surgery involved moderate hypothermia (27-28°C) for an average of 157 ± 49 minutes. Vancomycin concentrations in serum decreased upon initiation of CPB, likely due to hemodilution, followed by an increase in concentrations

after aortic clamping. Pharmacokinetic analysis demonstrated that the CL_R of vancomycin decreased with hypothermia during CPB; however this was not significant. The changes in vancomycin serum concentrations strongly correlated with the changes in body temperature ($r=0.92$). This study was also performed in patients undergoing CPB surgery treated with netilmicin and results demonstrated a decrease in netilmicin serum concentrations upon initiation of CPB. Netilmicin CL_R decreased during CPB compared to pre-CPB, however these results did not demonstrate significance. These two studies provide evidence that Cl_R of antimicrobial agents is decreased during hypothermia due to a reduction in cardiac output in CPB patients, leading to subsequent decreases in glomerular filtration rate.

All the studies presented in this section investigated the effects of hypothermia during cooling on drug disposition and metabolism; thereby providing evidence that hypothermia alters the pharmacokinetics of multiple drugs clinically utilized in CPB, TBI, and CA. Three major conclusions can be drawn from these studies. First, hypothermia affects multiple routes of drug elimination. For instance, hypothermia decreases Cl_H , specifically CYP450-mediated metabolism. Hypothermia also decreases biliary and renal excretion, both of which can alter the pharmacokinetics of drugs eliminated via these pathways. Second, alterations in the disposition of medications are specific to the disease for which hypothermia is being employed as is evidence by the findings in CPB since there are multiple factors affecting drug disposition in these patients (Lunn, Stanley et al. 1979; Koska, Romagnoli et al. 1981; Koren, Barker et al. 1987). This points to the difficulty in evaluating the effects of hypothermia on drug disposition and metabolism using certain models of injury that are more complicated; however they illustrate important insight into the complexity of these patients and the multiple factors which can affect the disposition of medications. Third, dosing nomograms are needed for a number of drugs used

in the ICU in order to optimize treatment in these patients. From these studies, we can conclude that mild to moderate hypothermia decreases the Cl_S of commonly used drugs between 7.1% and 22.3% per degree Celsius from the Cl_S and 37°C. Although this general reduction in Cl_H is observed, detailed studies of specific classes of medications used in the ICU are lacking. In addition mechanistic studies are needed to determine how hypothermia affects the CYP450 enzyme system.

1.4.3 Mechanisms of the Effects of Therapeutic Hypothermia During Cooling on CYP450-Mediated Metabolism

Hypothermia alters multiple aspects of enzymatic function (Somero 1975; Jaenicke 1991; Zachariassen 1991; Somero 2003). A model has been developed which shows that hypothermia can also decrease the substrate binding ability of multiple enzymes (Somero 2003). A potential mechanism behind the hypothermia-induced effects on CYP450-mediated metabolism is through changes in the binding pocket conformation. Hypothermia can decrease the affinity of the CYP450 for specific substrates (McAllister and Tan 1980; Tortorici, Kochanek et al. 2006). The effects of hypothermia during cooling on drug metabolism may also be mediated via a decrease rate of redox reactions performed by the CYP450 enzyme. Other potential mechanisms of hypothermia-induced alterations in CYP450 functionality include; changes in NADPH P450 reductase activity, cytochrome b5 activity, or the affinity of O₂ for the ferric iron, as well as changes in the lipid membrane fluidity. Although these proposed mechanisms lend some insight, careful mechanistic studies at clinically relevant temperatures are needed to confirm and/or refute these theoretical alterations in enzyme function.

Despite the wealth of data demonstrating significant alterations in drug disposition during therapeutic hypothermia, drug dosing is not typically altered in patients receiving hypothermia. In the case of CPB, the short duration of hypothermia is unlikely to produce significant drug toxicity due to the acute changes in disposition, with the exceptions of depth of anesthesia as is evident in the above mentioned studies. However, the recent use of therapeutic hypothermia in patients after CA, TBI, and stroke with durations ranging from 12 to 48 hours, is likely to produce significant alterations in drug concentrations and potentially increase the toxicity of medications commonly employed in this patient population. Currently, limited information is available about the magnitude of the alterations with respect to specific drugs and only a few studies provide recommendations for dosage adjustment during the acute phase of hypothermia. Future studies are needed to delineate the alterations and to develop guidelines for dosage adjustment in patients undergoing hypothermia. As will become evident in the remainder of this chapter, the changes in drug disposition due to hypothermia are not only limited to the "during cooling" effects on drug metabolism and elimination. Therapeutic hypothermia has been shown to alter drug efficacy and potency. Furthermore, therapeutic hypothermia also alters the pathogenesis of disease, thereby, altering the regulation of drug metabolizing gene expression in response to a given disease in the chronic (post-rewarming) phase. As is detailed below, these effects on drug response and the post-warming regulatory effects have been demonstrated in clinical and animal models, and require further study to determine the effects and implications of these alterations in patients receiving therapeutic hypothermia.

1.5 EFFECTS OF THERAPEUTIC HYPOTHERMIA ON DRUG EFFICACY AND POTENCY

We have reviewed the effects of therapeutic hypothermia on drug disposition and metabolism during cooling; however, there is also literature which has investigated the effects of hypothermia on drug response, particularly the potency and efficacy of commonly used drugs (Puig, Warner et al. 1987; Heier, Caldwell et al. 1991; Heier, Caldwell et al. 1994). The following section will review these articles and provide some mechanistic insight into these changes.

Morphine

Morphine is one of the most commonly used opioids and anesthetic adjuncts in clinical practice and is primarily metabolized by hepatic glucuronidation. Puig *et al.* demonstrated that the potency of morphine was decreased at 30°C compared to 37°C in the guinea-pig ileum preparation. This study investigated how changes in temperature would affect the interaction of morphine with its specific receptors in the electrically stimulated myenteric plexus-longitudinal muscle guinea-pig ileum. (Puig, Warner et al. 1987) Opioid receptor binding leads to a decrease in the ganglionic release of acetylcholine, thereby, exerting the drug response. At 30°C; morphine was one-fifth as potent compared to 37°C, as the IC₅₀ was significantly increased, 8.8 X 10⁻⁸ mol/L at 37°C vs. 41 X 10⁻⁸ mol/L at 30°C. There was also a large decrease in the affinity of morphine for its receptor as is evident by a six-fold increase in the dissociation constant at 30°C (45.5 ± 15 x 10⁻⁷ mol/L) compared to 37°C (8.3 ± 2.45 x 10⁻⁷ mol/L). This change in potency of morphine could be related to a hypothermia-mediated change in the dissociation constant for the *mu* receptor. Although hypothermia could decrease the metabolism

of morphine through glucuronidation, if hypothermia decreases the pharmacodynamic response, higher levels may be necessary to achieve an adequate response. This suggests that the dose response curve of morphine may be shifted, which indicates a change in drug response, however further research is needed to fully elucidate these changes. An important general axiom of drug therapy during mild or moderate hypothermia may be drawn from this study, suggesting that clinicians must carefully select drug with the lowest possible toxicity for use with hypothermia since higher concentrations may be needed to achieve a therapeutic effect.

Vecuronium

However, not all drugs show reduced potency with mild hypothermia. Heier et al. investigated the effects of mild hypothermia on neuromuscular junction sensitivity to vecuronium and determined the potency and efficacy (Heier, Caldwell et al. 1994). Five subjects were cooled to $\sim 34^{\circ}\text{C}$ and five subjects were normothermic $\sim 37^{\circ}\text{C}$. Vecuronium was administered as an IV infusion at a rate of $3\ \mu\text{g}/\text{kg}/\text{min}$ for 10 min. Although there was an increase in the duration of action of vecuronium at 34°C , there were no differences between hypothermic and normothermic subjects as measured by the steady-state concentration producing 50% depression of twitch tension on the train-of-four test. The equilibrium rate constants of vecuronium between the plasma and the neuromuscular junction were $0.27 \pm 0.14/\text{min}$ at 34.4°C and $0.26 \pm 0.11/\text{min}$ at 36.8°C . Thus, hypothermia-mediated increases in the duration of action of vecuronium are not due to changes in drug potency and efficacy, but more likely due to a change in the pharmacokinetics of vecuronium. Consequently, it would be important to determine which drugs used in the ICU exhibit reduced potency with hypothermia, since these hypothermia-mediated effects seem to show substantial variability between drugs. We believe

that this is an important area for future research that would be critical to the development of optimal dosing recommendations for each of the agents.

1.6 THE EFFECTS OF HYPOTHERMIA ON DRUG DISPOSITION AND METABOLISM AFTER RE-WARMING

Figure 5 demonstrates the stages of the effects of hypothermia on drug metabolism and disposition that we have identified after an injury, such as CA, TBI or stroke. We believe that not only is hypothermia producing alterations in drug disposition during cooling; there could be significant changes during each of the phases we have identified after cooling. This section examines these effects of hypothermia on the metabolism of drugs commonly used in the ICU.

The most complete clinical study investigating both the effects of hypothermia during cooling and after re-warming on drug disposition and metabolism evaluated phenytoin pharmacokinetics during and post-hypothermia in TBI patients. Mild hypothermia was induced to a target temperature at 34°C and maintained for 4 hrs. Fourteen patients were cooled to 34°C and phenytoin was given as a loading bolus dose. Patients were also dosed with phenytoin 7-8 days after injury when core temperature was normothermic (36°C). Hypothermia produced increases in AUC and a decrease in the Cl_s of phenytoin vs. normothermia. After re-warming, (7-8 days) phenytoin pharmacokinetic parameters were normalized to pre-hypothermia levels.

INJURY

Cardiac Arrest

TBI, Stroke

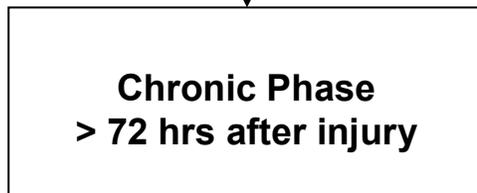


Figure 5: Phases of hypothermia-induced changes after injury

Russell *et al.* performed a study investigating the effects of CPB on propofol concentrations in ten male patients (Russell, Wright et al. 1989). Propofol was administered as continuous infusion at a rate of 10 mg/kg/hour and then decreased to 3 mg/kg/hour for approximately 250 minutes. Hypothermia was induced (25°C-27°C) for approximately 50 minutes followed by re-warming. Propofol concentrations were higher during the hypothermic period. Once re-warming was started propofol blood concentrations declined to pre-hypothermia/CPB concentrations. This study demonstrated that propofol concentrations are returned to normal levels once normothermia was reached.

Collectively, these studies show that plasma concentrations of the drugs studied return to pre-hypothermia levels once the patient's core temperature is returned to normal. Though future trials are needed to fully elucidate the mechanisms behind the effects of hypothermia on drug disposition and metabolism, these studies can provide the clinician with an insight into the time-course of hypothermia-induced alterations.

1.6.1 Mechanisms of the Effects of Therapeutic Hypothermia after Re-warming on CYP450-Mediated Metabolism

Data in Chapter 3 demonstrates that CA produces a decrease in CYP2E1-mediated metabolism of chlorzoxazone 24 hrs after insult, in the sub-acute phase after injury (Tortorici, Kochanek et al. 2006). TBI has also been shown to produce an initial decrease in CYP450 functionality followed by a hyper-metabolic state (Boucher and Hanes 1998; Harbrecht, Frye et al. 2005). As mentioned in the previous section, the studies investigating the effects of hypothermia after re-warming on the disposition of drugs during ICU-relevant insults demonstrate that drug concentrations return to normal (Russell, Wright et al. 1989; Gruber, Laussen et al. 2001; Iida,

Nishi et al. 2001). This indicates that hypothermia is producing a protective effect by mitigation of cellular damage after injury with normalization of otherwise altered drug metabolism.

Cardiac arrest and TBI produce increases in pro-inflammatory cytokines (Boucher and Hanes 1998; Adrie, Adib-Conquy et al. 2002; Tortorici, Kochanek et al. 2006). Hypothermia reduces pro-inflammatory cytokines, including interleukin-6 (IL-6) and interleukin-1 β , after CA and TBI in both experimental and clinical models (Marion, Penrod et al. 1997; Aibiki, Maekawa et al. 1999; Truettner, Suzuki et al. 2005; Tortorici, Kochanek et al. 2006). These pro-inflammatory cytokines decrease both the expression and activity of multiple CYP450 isoforms (Abdel-Razzak, Loyer et al. 1993; Projean, Dautrey et al. 2005). Thus, mild hypothermia may reduce tissue damage; thereby normalizing drug metabolism in the sub-acute phase after injury. This suggests that after various insults such as CA, benefits of mild hypothermia may favorably alter the natural course of disease, normalizing drug metabolism.

1.7 CONCLUSION

This chapter has demonstrated that therapeutic hypothermia dramatically affects drug disposition involving a complex interplay between the effects on drug metabolism, elimination, and response during cooling and after re-warming. For example, the studies outlined in this Chapter indicate that during cooling, drug metabolism is dramatically decreased. This may lead to the conclusion that the dose of the drug should be reduced in order to avoid toxicity. However, the effects of hypothermia on drug response are less predictable; demonstrating both reduced potency or no change in the potency depending on drug class. Overall, the effects of hypothermia during cooling may suggest a narrowing of the therapeutic index of multiple drugs used in the ICU.

After re-warming drug metabolism is returned to normal. Based on these variable time-dependent changes, it is essential that intensivists are cognizant of the potential changes in drug disposition during and after cooling, monitor drug levels when possible, and monitor outcomes (i.e. depth of neuromuscular blockade, sedation scores) during and after hypothermia. The value of other modalities such as the bispectral index (BIS) should be studied during hypothermia.

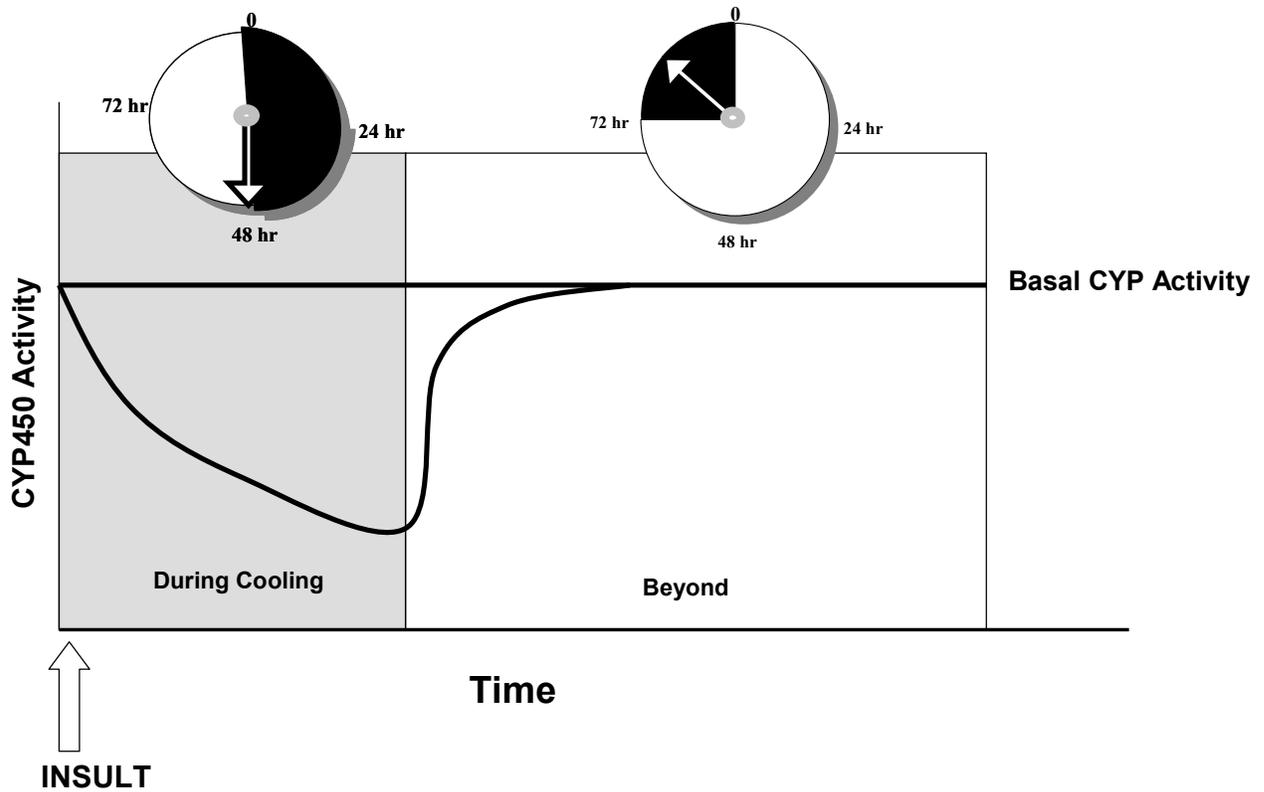


Figure 6: Time-course of hypothermia-induced effects on cytochrome-P450 activity

Therapeutic hypothermia (solid line) demonstrates a decrease in cytochrom-P450 activity followed by a return to normal activity after re-warming.

The ability of critical care clinicians (including physicians, nurses, and pharmacists) to identify potential hypothermia-induced alterations in drug disposition provides multiple

opportunities for future research to systematically evaluate these effects. These included clinical trials with the primary objective to identify hypothermia-mediated changes in the pharmacokinetics of commonly used drugs in the ICU. Furthermore, in current and future clinical trials investigating the beneficial effects of therapeutic hypothermia in insults such as stroke and TBI, it will be essential to also explore the pharmacokinetics of the drugs used during these studies to determine if certain drugs are beneficial or harmful during hypothermia.

Collectively, the studies outlined throughout this review can provide us with a theoretical time-course of alterations on drug disposition and metabolism (specifically CYP450-mediated) produced by therapeutic hypothermia. As depicted in **Figure 6**, hypothermia produces a dramatic decrease in CYP450 activity during the period of cooling, followed by a rebound of CYP450 activity once core temperature is returned to normothermia. Future studies are needed \ in order to provide specific dosing nomograms for clinically utilized medications during hypothermia. These data imply that clinically significant hypothermia-drug interactions are a likely factor to be considered in the therapeutic course of these patients.

The work presented throughout this dissertation aims to systematically investigate the effect of therapeutic hypothermia on drug metabolism in experimental and clinical models. As presented throughout this Chapter, hypothermia can affect metabolism, absorption, elimination, distribution and response of many drugs. We have chosen to focus this work on the effects of cooling on the CYP450 enzyme system. Many of the historical studies have demonstrated that hypothermic temperatures affect drugs metabolized by CYP450's during cooling; however there has never been a systematic evaluation to elucidate the mechanisms behind these changes. In addition we have focused our work in an animal model of cardiac arrest, since the use of mild

hypothermia in patients experiencing out-of-hospital cardiac arrest has been shown to decrease mortality and morbidity.

Based on this, we hypothesize that therapeutic hypothermia produces time-dependent alterations in CYP450 activity resulting in significant alterations in the disposition of medications used in critically ill patients. In order to evaluate this hypothesis the following specific aims have been proposed by chapter;

Specific Aim 1: Determine the *in vivo* effect of moderate hypothermia on the pharmacokinetics of the CYP2E1 substrate, chlorzoxazone, in a rat model of cardiac arrest.

Specific Aim 2: Determine the effects of cardiac arrest and moderate hypothermia in a rat model on the functional activity and expression of CYP3A2 and CYP2E1 and examine the regulation of these isoforms after injury.

Specific Aim 3: Evaluate the effects of mild and moderate hypothermia on the *in vitro* enzyme kinetics of CYP3A4 and CYP2E1 in human hepatic microsomes and therefore predict *in vivo* changes in humans.

Specific Aim 4: Investigate the effects of mild hypothermia on midazolam pharmacokinetics in normal healthy subjects.

The body of work presented in this dissertation begins to answer these specific aims by evaluating the effect of mild to moderate hypothermia in a systematic method via utilizing

multiple animal models, *in vitro/in vivo* correlations, and a model of hypothermia in normal healthy volunteers.

2.0 CHAPTER 2

Therapeutic Hypothermia Induced Pharmacokinetic Alterations on CYP2E1 Chlorzoxazone Mediated Metabolism in a Cardiac Arrest Rat Model

[Tortorici, MA, Kochanek PM, Bies RR, and Poloyac SM. Therapeutic Hypothermia Induced Pharmacokinetic Alterations on CYP2E1 Chlorzoxazone Mediated Metabolism in a Cardiac Arrest Rat Model. *Crit Care Med* 2006;34(3)785-791.]

INTRODUCTION

Chapter 1 reviewed the beneficial effects of therapeutic hypothermia in patients experiencing out-of-hospital cardiac arrest and other ischemic injuries, such as TBI and stroke. Clinical implementation of hypothermia has increased as a result of two landmark clinical trials that showed that the use of mild hypothermia as a therapy in out-of-hospital CA patients decreased morbidity and mortality and improved survival (Bernard, Gray et al. 2002; Group 2002). In addition to therapeutic hypothermia, patients experiencing CA are subjected to an extensive pharmacotherapeutic regimen. The majority of these agents used are eliminated via hepatic metabolic pathways, more specifically the cytochrome P450 (CYP450) system. As described in detail in Chapter 1, prior clinical and *in vivo* animal studies have suggested that therapeutic hypothermia alters the pharmacokinetics of medications metabolized by the CYP450 system, however a thorough mechanistic evaluation of the effects of hypothermia on the CYP450 system has not been reported (Koren, Barker et al. 1985; Koren, Barker et al. 1987; Leslie, Sessler et al. 1995; Caldwell, Heier et al. 2000; Iida, Nishi et al. 2001; Fukuoka, Aibiki et al. 2004).

With the increase in clinical implementation and the paucity of information that exists on the effects of therapeutic hypothermia on drug metabolism, we set out to mechanistically examine the effects of therapeutic hypothermia on CYP2E1-mediated metabolism in a clinically relevant CA rat model. As previously mentioned, CYP2E1 plays a major role in the metabolism of multiple compounds such as ethanol, isoniazid, and theophylline, as well as the metabolism of arachidonic acid (Lieber 1997). Chlorzoxazone (CZN) is a drug that is exclusively metabolized

by CYP2E1 and is used as a probe substrate to assess the activity of this enzyme. The systemic clearance (Cl_s) of CZN approximates the intrinsic clearance (Cl_{int}) by the CYP2E1 enzyme. The Cl_{int} is defined as the inherent ability of the enzyme to remove drug from the body. Based on this, the objectives of this study were to 1) evaluate the effects of therapeutic hypothermia on the pharmacokinetics of CZN during the hypothermic period in a rat model of CA and 2) to identify the mechanisms underlying these alterations in the Cl_s , by evaluating the effects of hypothermia on CZN plasma protein binding and the Cl_{int} of CYP2E1, using *in vitro* techniques.

2.1 METHODS

2.1.1 Materials and Chemicals

Male Sprague-Dawley rats (body weight, 300-400 g) were purchased from Hilltop Laboratories (Scottsdale, PA). Chlorzoxazone (CZN), 6-hydroxychlorzoxazone (6-OH CZN), and umbelliferone, were purchased from Sigma Aldrich (St Louis, MO.) Organic solvents were purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals were purchased from Sigma Aldrich unless otherwise specified.

2.1.2 Animal Cardiac Arrest and Hypothermia Protocol

Rats were allowed free access to food and water before and after surgery. The University of Pittsburgh Animal Care and Use Committee approved all studies. Sterile technique was used for all surgical procedures.

CA was conducted as previously described (Fink, Alexander et al. 2004; Katz, Young et al. 2004). Rats were anesthetized with 3% halothane via a nose cone, endotracheally intubated, and mechanically ventilated to maintain PaCO₂ between 35 to 45 mmHg. Halothane concentration was then reduced to between 0.5 to 1%. The right femoral artery was cannulated for measurement of mean arterial pressure and the femoral veins were cannulated bilaterally for drug administration and blood sampling. Neuromuscular blockade was induced with vecuronium 2 mg/kg (IV). A rectal probe was inserted for body temperature measurements. After preparative surgery, halothane was washed out with 100% O₂ for 3 minutes and room air for 2 minutes. Rats were randomized to receive either, CA with normothermia (CA Normothermia), or CA with hypothermia (CA Hypothermia), n=6. Animals received another vecuronium 1 mg/kg IV dose and CA was initiated by asphyxiation via disconnection of the mechanical ventilation. Asphyxial arrest was conducted for 8 minutes, with 5 minutes of asystole, confirmed by ECG trace as previously described (Fink, Alexander et al. 2004). Resuscitation was started by reconnecting the rat to the ventilator, administering epinephrine (0.005 mg/kg), IV and bicarbonate (1mEq/kg), IV and performing manual chest compressions for about one minute until restoration of spontaneous circulation (ROSC). Halothane was not resumed after asphyxiation based on prior documentation of a burst-suppression pattern on EEG during early recovery (Fink, Alexander et al. 2004). Arterial blood gases and glucose measurements were taken prior to asphyxia and at the end of the re-warming phase. Acidosis was corrected via bicarbonate replacement to maintain pH at 7.4.

After ROSC, rats were either maintained at 37°C normothermia or body temperature was decreased to 30°C hypothermia over 15 minutes via surface cooling. Once body temperature had stabilized, CZN (15mg/kg) was administered IV as a single dose via the right femoral vein.

CZN was given at approximately 35 minutes after the end of asphyxial arrest and 45 minutes after halothane washout. Blood samples (0.3 ml) were obtained via the left femoral venous cannula with a heparinized syringe at 0, 10, 20, 30, 60, 90, 120, 150, and 165 minutes after drug administration and urine was obtained by collection of the first urine sample upon recovery from neuromuscular blockade. Rats remained hypothermic over the entire 3 hour period after temperature stabilization, during drug administration, and blood collection. Intensive care management was performed in all animals and included as needed doses of vecuronium, bicarbonate, and butorphanol. Re-warming was conducted over 1 hour and rats were gradually weaned from mechanical ventilation, extubated, recovered, and then returned to their cages. Urine was collected at the end of re-warming by a collection dish placed under the bladder as rats recovered from neuromuscular blockade. Rats were sacrificed 24 hours post-insult via decapitation and liver, kidney, and brain tissue were retrieved for a separate study.

2.1.3 Chlorzoxazone Analysis

Plasma concentrations of CZN were measured by a modified HPLC method described by Rockich *et al* (Rockich and Blouin 1999). Briefly, 100 μ l of plasma or urine from each sample was added to 900 μ l of 10 mM sodium acetate buffer (pH=4.5) and incubated with 500 μ l of β -glucuronidase (1000 units) for 3 hours in a 37°C circulating water bath in order to cleave the glucuronide from the 6-hydroxylated metabolite in the urine and plasma. Internal standard, umbelliferone, was added to each plasma and urine sample and then extracted with 5 ml of diethyl ether. The organic phase was transferred and evaporated to dryness under a stream of nitrogen. Samples were then reconstituted with 200 μ l of mobile phase A, consisting of 78% of

a 0.25% acetic acid solution and 22% acetonitrile, and 20 μ l was injected onto the HPLC system. Mobile phase B consisted of 95% acetonitrile and 5% of a 0.25% acetic acid solution and a gradient was established. CZN, umbelliferone, and 6-OH CZN were separated using a Nova Pak C-18 reverse phase (5 μ m, 3.9 X 150mm) column (Waters, Millford, MA). The HPLC system consisted of a Waters 2695 with a 2487 UV detector, set at 287 nm for CZN and 296 nm for 6-OH CZN. The ratio of CZN or 6-OH CZN to umbelliferone was used for quantitative analysis. The C.V. was less than 10% for this assay

2.1.4 Protein Binding Analysis

Protein binding of CZN in rat plasma was determined by the ultrafiltration technique as reported by Rockich and Blouin (Rockich and Blouin 1999). Each sample (n=4) consisted of 1.5 ml of control rat plasma, spiked with a stock solution of CZN, to give a final concentration of 10 μ g/ml. The samples were incubated for 15 minutes at 37°C or 30°C, and 1-ml aliquots were placed into an ultrafiltration device (Amicon, Beverly, MA) equipped with an ultrafiltration membrane. The remaining 500 μ l was analyzed for total CZN concentration. The samples were then centrifuged at 3200 rpm at 37°C or 30°C, respectively, for 30 minutes or until 250 μ l of filtrate was collected. Concentrations of CZN in the filtrate, the 1-ml aliquot, and the remaining original 1.5 ml plasma sample were determined by the HPLC method described above. The fraction of unbound (fu) CZN was calculated by dividing the concentration in the filtrate/ concentration in the original plasma sample, which represents unbound concentration/ total concentration.

2.1.5 CYP2E1 Enzyme Kinetic Analysis

Liver tissue was collected from 4 untreated rats and microsomes were prepared as previously described by Rockich and Blouin (Rockich and Blouin 1999). All microsomal incubations contained 400 µg microsomal protein and 1 mM NADPH. CZN was added to microsomal samples in increasing concentrations of 50, 75, 100, 200, 300, 400, and 500 µM. These samples (n=4 per temperature group) were either incubated for 20 minutes at 37°C or 30°C in a circulating water bath. The reaction was stopped by adding 50 µl of 42.5% o-phosphoric acid, followed by the addition of the internal standard, umbelliferone. Samples were analyzed as stated above.

2.1.6 Pharmacokinetic and Enzyme Kinetic Analysis

The plasma concentration-time curves for CZN were fit to a one-compartment model and were analyzed using the computer program WinNonlin Pro 4.1 (Pharsight Corp, Mountain View, CA).

$$C_t = C_o e^{-kt} \quad (1)$$

The area under the plasma concentration-time curve (AUC) for CZN was determined by the trapezoidal rule with extrapolation to infinity, linear up and logarithmic down. The Cl_s was calculated as dose/area under the plasma concentration-time curve. The half-life was calculated using 0.693 divided by the elimination rate constant (K). Visual inspection and sum of squares residuals were determined for model fitness and weighting.

The effect of incubation temperature on CYP2E1 enzyme kinetics was analyzed in order to investigate the mechanism of the alterations of the clearance values as observed in the CA Hypothermia versus CA Normothermia groups. A formal Michaelis-Menten kinetic analysis

was performed from the formation rates using WinNonlin (Pharsight Corp, Mountain View, CA). Maximum velocity (V_{max}) and the concentration Michaelis-Menten constant (K_m) were calculated via non-linear regression of the raw data. The data was then linearized for visual inspection, via an Eadie-Hofstee plot.

The following equation was used for analysis:

$$v = V_{max} * [S] / (K_m + [S]) \quad (2)$$

where v is the velocity and $[S]$ is the substrate concentration.

The Cl_{int} (ml/min) was calculated using the equation:

$$Cl_{int} = (V_{max}/K_m) * \text{mg microsomal protein} \quad (3)$$

The *in vitro* Cl_{int} was used to estimate the *in vivo* Cl_s in a rat as described by Tong *et al* (Tong, Abbott et al. 2001). The following equation explains the predicted *in vivo* parameters from *in vitro* estimates:

$$Cl_{int}' = Cl_{int \text{ in vitro}} * (\text{mg microsomal protein/g of liver}) * (\text{liver weight g/ body weight kg}) * (\text{ml incubation/ mg microsomal protein}) \quad (4)$$

Where Cl_{int}' is the estimated *in vivo* intrinsic clearance. The estimated Cl_s was then calculated by multiplying the Cl_{int}' and the f_u . All results are given as means \pm S.D.

2.1.7 Statistical Analysis

For comparisons of physiologic parameters over time a two-way ANOVA for repeated measures with a Bonferroni correction was used. For individual comparisons a Student's t-test was utilized, assuming equal variances, to compare the CA Normothermia group and the CA Hypothermia group. We considered $p < 0.05$ to be statistically significant. Data are presented as

mean \pm S.D. All analyses were conducted using the software program Prism (GraphPad Software, San Diego, CA).

2.2 RESULTS

2.2.1 Time-Course of Hypothermia in Cardiac Arrest Treated Rats

As depicted in **Figure 7**, rats were either maintained at 37°C or were cooled to 30°C over 15 minutes. Hypothermia was maintained for 3 hours followed by 1 hour of re-warming.

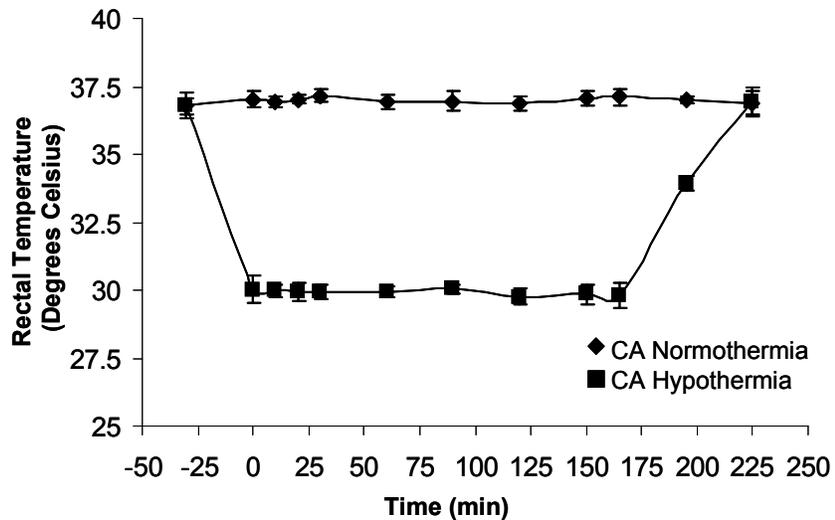


Figure 7: Time-course of temperature in the rat

2.2.2 Physiological Parameters in Cardiac Arrest Rats

Physiological parameters (MABP, blood pH, PaO₂, PaCO₂, and glucose) were measured at two time points (**Table 3**). The pre-CA/hypothermia measurements were drawn after preparative

surgery and immediately prior to the initiation of CA. The post-CA/hypothermia measurements were drawn after 3 hours of hypothermia and 1 hour of re-warming. The data demonstrates that pre-CA/hypothermia showed no significant alterations in physiologic parameters between groups. However, post-CA/hypothermia parameters demonstrate that both groups have an anticipated increase in PaO₂ and decrease in PaCO₂, when compared to pre-CA/hypothermia parameters (**p<0.01) (Fink, Alexander et al. 2004). All other parameters demonstrated no statistical differences.

Table 3: Physiological Parameters in Cardiac Arrest and Hypothermia Rats

	Experimental Group	Pre-CA/Hypothermia	Post-CA/Hypothermia
MABP (mmHg)	CA Normothermia	101.17 ± 9.60	114.83 ± 10.23
	CA Hypothermia	104.33 ± 10.13	104.17 ± 27.46
pH	CA Normothermia	7.39 ± 0.02	7.39 ± 0.04
	CA Hypothermia	7.38 ± 0.02	7.41 ± 0.07
PaCO ₂ (mmHg)	CA Normothermia	38.17 ± 2.64	29.58 ± 4.13 ^{***}
	CA Hypothermia	40.00 ± 0.63	25.50 ± 3.89 ^{***}
PaO ₂ (mmHg)	CA Normothermia	224.83 ± 32.37	414.67 ± 49.99 ^{***}
	CA Hypothermia	227.50 ± 46.12	491.33 ± 51.15 ^{***}
Glucose (mg/dL)	CA Normothermia	119.83 ± 18.42	116.00 ± 19.37
	CA Hypothermia	121.00 ± 10.58	146.67 ± 28.79

Abbreviations: MABP, mean arterial blood pressure; PaCO₂, pressure of carbon dioxide; PaO₂, pressure of oxygen

2.2.3 Effect of Hypothermia on the Pharmacokinetics of Chlorzoxazone in Cardiac Arrest Treated Rats

The linear plot of CZN concentration-versus-time profile followed a simple mono-exponential decline for both the CA Normothermia and CA Hypothermia groups. As depicted in **Figure 8, Panel A**, CA Hypothermia significantly altered the plasma concentration-versus-time profile compared to the CA Normothermia group. **Table 4** depicts the pharmacokinetic parameter results and demonstrates that CA Hypothermia significantly altered a number of pharmacokinetic parameters, such as Cl_s , K , and V_d . Cl_s in the CA Hypothermia group (0.580 ± 0.37 ml/min) was decreased vs. the CA Normothermia group (1.26 ± 0.34 ml/min; $**p < 0.01$). The elimination rate constant (K) in the CA Hypothermia group (0.00210 ± 0.0015 min⁻¹) was decreased vs. the CA Normothermia group (0.00614 ± 0.0019 min⁻¹; $**p < 0.01$). The volume of distribution (V_d) in the CA Hypothermia group (288 ± 42 ml) was increased vs. the CA Normothermia group (211 ± 35 ml; $**p < 0.01$).

Figure 8, Panel B, depicts the urinary excretion rate of 6-OH CZN over 165 minutes for both groups. This data shows the rate of excretion of 6-OH CZN in the CA Hypothermia group is 0.255 ± 0.24 µg/min vs. 1.05 ± 0.74 µg/min for the CA Normothermia group; $*p < 0.05$.

2.2.4 Effect of Temperature on Protein Binding of Chlorzoxazone

The total, bound, and unbound concentrations were analyzed. The f_u at 37°C was 0.0458 ± 0.0061 compared to 30°C which was 0.0422 ± 0.0041 , demonstrating no statistical difference as depicted in **Figure 9**.

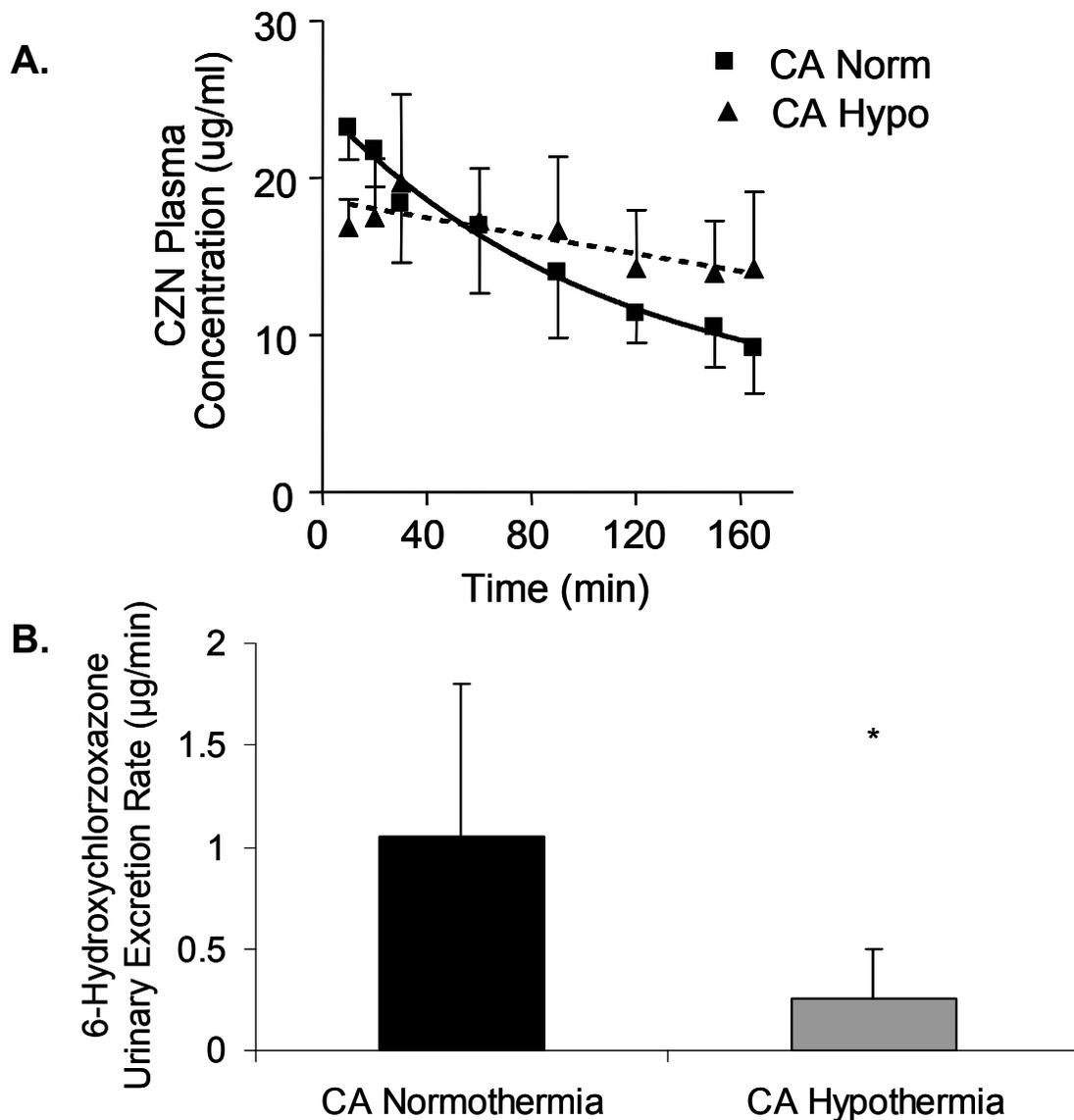


Figure 8: Pharmacokinetic profile of chlorzoxazone and urinary excretion rate of 6-hydroxychlorzoxazone.

Panel A depicts the linear plot of the chlorzoxazone plasma concentrations-versus-time curve in cardiac arrest normothermia vs. cardiac arrest hypothermia treated rats after treated with IV bolus dose of chlorzoxazone (15mg/kg). Each point represents the mean \pm S.D.; n=6. Panel B depicts the urinary excretion rate of 6-hydroxychlorzoxazone in both groups. Data shows a significant decrease in cardiac arrest hypothermia rats ($0.255 \pm 0.24 \mu\text{g}/\text{min}$) vs. cardiac arrest normothermia ($1.05 \pm 0.74 \mu\text{g}/\text{min}$) $p < 0.05$.

Table 4: Pharmacokinetic parameters in cardiac arrest treated rats

Parameters	CA Normothermia	CA Hypothermia
Cl _s (ml/min)	1.26 ± 0.34	0.58 ± 0.37 **
t _{1/2} (min)	125.36 ± 48.92	620.98 ± 597.22
V _d (ml)	211.17 ± 34.76	288.12 ± 42.25 **
K (min ⁻¹)	0.0061 ± 0.0013	0.0021 ± 0.0015 **
AUC (min*ug)/ml/kg	12.63 ± 3.77	29.94 ± 18.3 *

Abbreviations: Cl_s, systemic clearance; V_d, volume of distribution; K, elimination rate constant; AUC, area under the plasma concentration-time curve.

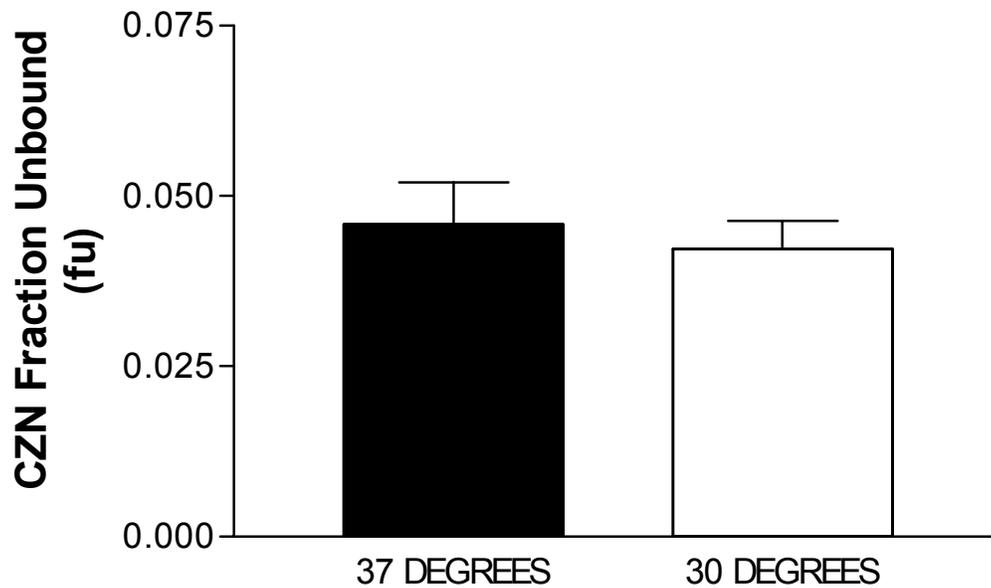


Figure 9: Protein binding of chlorzoxozone at 37°C and 30°C.

Table 5: Michaelis-menten enzyme kinetic parameter estimates in rat liver microsomes

	37 DEGREES	30 DEGREES
Vmax (nmol/mg/min)	2.27 ± 0.22	2.64 ± 0.48
Km (uM)	255.53 ± 52.03	551.6 ± 149.91 **
Clint (ml/min/mg protein)	0.0089 ± 0.0017	0.0050 ± 0.0004 **
Cl_s estimated (ml/min)	2.10 ± 0.39	1.19 ± 0.21 **

Abbreviations: Vmax, maximal velocity; Km, Michaelis-menten constant; Clint, intrinsic clearance; Cl_s, systemic clearance

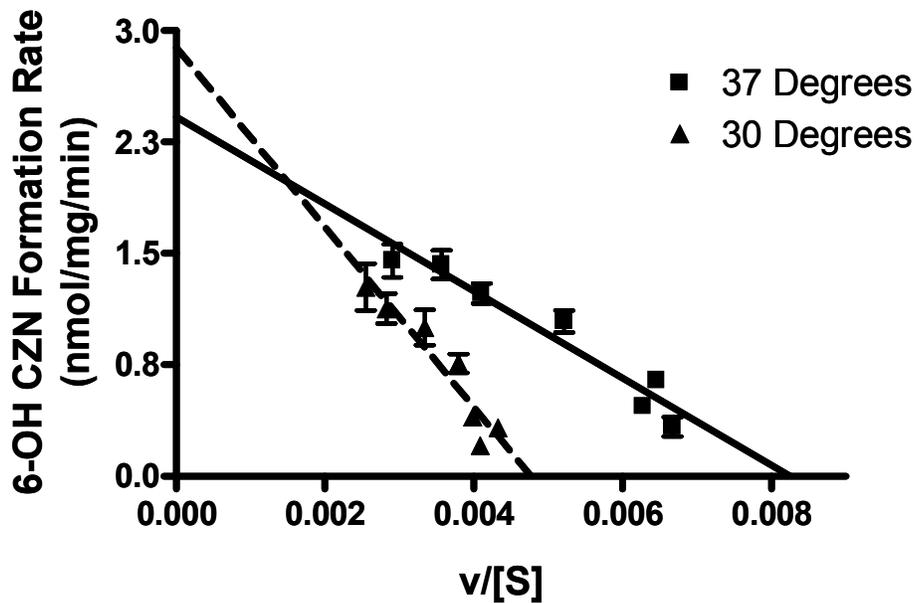


Figure 10: Eadie-hofstee plot of enzyme kinetic analysis of CYP2E1

Constructed from the non-linear regression analysis of raw data reported in Table 5. The difference in Km can be observed by the difference in slopes of the 2 lines.

2.2.5 Effect of Temperature on CYP2E1 Enzyme Kinetics

There were no significant differences in CYP2E1 V_{\max} for 37°C (2.27 ± 0.22 nmol/mg/min) versus 30°C (2.65 ± 0.48 nmol/mg/min), **Table 5**. However, when the K_m was analyzed, an increase was observed between the 30°C group (551 ± 150 μ M) vs. the 37°C group (255 ± 52 μ M; $**p < 0.01$). For visual inspection, an Eadie-Hofstee plot was constructed (**Figure 10**) and the difference in the slope of the lines between the 37°C and 30°C groups represents the changes observed in the K_m values. The y-intercept represents the V_{\max} of each group and shows no statistical differences.

The Cl_{int} was decreased in the hypothermia group, 37°C (0.00890 ± 0.0017 ml/min/mg protein) and 30°C (0.00500 ± 0.00040 ml/min/mg protein; $**p < 0.01$), as depicted in **Table 5**. An *in vitro/in vivo* correlation was performed using equations 3 and 4 (Tong, Abbott et al. 2001). Microsomal protein content has been previously estimated to be 40 mg protein/ g of liver. The average weight of liver in our rats was estimated to be 47 g/ kg body weight (Bachmann and Sarver 1996). These predicted *in vivo* Cl_{int} parameters were multiplied by the f_u of CZN in order to predict an *in vivo* Cl_s of 2.10 ± 0.39 ml/min at 37°C incubation temperature and 1.19 ± 0.21 ml/min at 30°C incubation temperature, shown in **Table 3**.

2.3 DISCUSSION

Our results demonstrate three novel findings. The first is that in a CA rat model, moderate hypothermia markedly alters the pharmacokinetics of the CYP2E1 probe drug, CZN. The second finding is that hypothermia treatment resulted in a decrease in Cl_s of CZN and this reduction is due to a decrease in the Cl_{int} of the CYP2E1 enzyme. The third finding is that the

reductions of Cl_{int} measured *in vitro* due to hypothermia, accurately predicted the magnitude of decrease observed in Cl_s of CZN in the CA rat model *in vivo*. These results represent the first in a series of designed experiments to identify certain changes in drug metabolizing enzymes in order to provide algorithms for dose adjustments of commonly used medications in the critical care patient receiving hypothermic therapy.

We showed a significant impact of moderate hypothermia on the pharmacokinetics of CZN after CA in rats treated with hypothermia versus normothermia. We observed reductions in the K_e , urine excretion rate, and the Cl_s , and observed an increase in the V_d . These effects have been substantiated by previous *in vivo* studies demonstrating the inhibition of hepatic drug elimination (Koren, Barker et al. 1985; Koren, Barker et al. 1987; Heier, Caldwell et al. 1991; Caldwell, Heier et al. 2000; Iida, Nishi et al. 2001; Michelsen, Holford et al. 2001; Statler, Alexander et al. 2003; Fukuoka, Aibiki et al. 2004). Fukuoka *et al.* showed a 5-fold increase in plasma concentrations of midazolam when the core temperature of brain-injured patients was maintained below 35°C (Fukuoka, Aibiki et al. 2004). Collectively, these studies show that hypothermia impacts the disposition of certain hepatically eliminated drugs, more specifically by decreasing the Cl_s .

Reductions in drug clearance by moderate hypothermia may represent a key toxicity that limits the putative benefit of this therapy. Statler *et al.* performed a study to evaluate the effects of hypothermia on lesion volume in TBI rats using fentanyl anesthesia (Statler, Alexander et al. 2003). Contrary to previous studies showing decreased lesion volume with hypothermia in the TBI isoflurane anesthesia rat, Statler *et al.* observed a larger lesion volume in the hypothermia group when fentanyl was used as the anesthetic agent. This study further showed that in the hypothermia group, there was an increase in serum concentrations of fentanyl versus

normothermia. The authors indicate that this increase in serum fentanyl concentrations could contribute to toxicity and may be the potential mechanism underlying the increase in lesion volume (Kofke, Garman et al. 1999); thereby, demonstrating a critical need for full knowledge of the adverse events of therapeutic hypothermia in order to optimize care after injury.

Our *in vivo* findings show a reduction in the Cl_s of CZN due to hypothermia in the CA model. Hypothermia affects the CYP450 system as reported in previous studies (Heier, Caldwell et al. 1991; Heier, Caldwell et al. 1994; Leslie, Sessler et al. 1995). A series of studies by Heier *et al.* demonstrated that the duration of action of vecuronium, metabolized by the CYP450 system, is greater than 3-fold longer in hypothermic (34.5°C) versus normothermic (36.5°C) surgical patients (Heier, Caldwell et al. 1991). Subsequent studies by Heier *et al.* showed that the changes in duration of action is due to pharmacokinetic alterations, specifically reductions in Cl_s , with no observed differences in the pharmacodynamic measures (Heier, Caldwell et al. 1994). These studies indicate that hypothermia alters the clearance of drugs through a reduction in hepatic elimination.

In order to elucidate the mechanism of these reductions, the parameters which dictate the Cl_s of CZN were further investigated. Hepatic clearance (Cl_H) of drugs with low clearance depend upon the f_u , and the Cl_{int} , as predicted by the well-stirred model of Cl_H (Pang and Rowland 1977; Pang and Rowland 1977; Pang and Rowland 1977). The Cl_{int} is a measure of the intrinsic ability of the enzyme to actively clear drug from the body and can be calculated by V_{max}/K_m . The temperature difference in this study had no effect on the protein binding of CZN and the V_{max} of the CYP2E1 enzyme, which was consistent with previous reports (Rockich and Blouin 1999; Tanaka 2001). However, at an incubation temperature of 30°C, K_m is significantly increased when compared to 37°C. The observed increase in K_m due to hypothermic conditions

is consistent with a study by McAllister *et al.* who demonstrated that when propranolol and verapamil were incubated at 26°C vs. 37°C, the K_m was significantly increased at the lower temperature (McAllister and Tan 1980). Collectively these results imply that moderate hypothermic temperatures decrease the CYP2E1 affinity for CZN, defining a key mechanism for the reduction of drug metabolism by this intervention.

The observed reductions in the Cl_s of the CA Hypothermia group can be explained by changes in the Cl_{int} of CYP2E1 due to hypothermia, using an *in vitro/in vivo* correlation analysis (Obach, Baxter et al. 1997). The *in vitro* Cl_{int} of each group showed a two-fold decrease in the microsomes incubated at 30°C versus 37°C. The magnitude of change in the predicted *in vivo* Cl_s between normothermia and hypothermia treated microsomes is approximately a 2-fold decrease from *in vitro* results. This implicates that the changes in the *in vitro* Cl_{int} can accurately predict the changes observed in the *in vivo* analysis.

We showed a significant increase in the V_d in the CA Hypothermia group compared to the CA Normothermia group. The V_d is a term which relates the amount of drug in the body to the concentration in the blood or the plasma. Hypothermic therapy increased the V_d in the CA Hypothermia group versus CA Normothermic controls, which has been observed for other medications (Fukuoka, Aibiki et al. 2004). A higher V_d indicates that the drug is more widely distributed out of the plasma and into the tissues. This could be due to multiple factors such as changes in plasma protein binding, global blood perfusion to organs, diffusion across membranes, or drug-tissue binding. We showed no difference in plasma protein binding at hypothermic temperatures and the other factors have never been investigated.

There are several possible limitations of our study. First, many of the pharmacokinetic parameters in the CA Normothermia group are different than historic control values, in naïve rats

(Rockich and Blouin 1999). These deviations are likely due to CA affects on CYP2E1-mediated metabolism of CZN. CA is a global ischemia/reperfusion injury. Reperfusion causes a systemic inflammatory response, resulting in large releases of cytokines and nitric oxide, both of which have been shown to affect CYP450-mediated metabolism (Abdel-Razzak, Loyer et al. 1993; Khatsenko 1998; 2000; Adrie, Adib-Conquy et al. 2002; Qi, Chaiyakit et al. 2004). The role of these mediators in our CA model has been evaluated as outlined in Chapter 3. Hepatic damage, due to CA, could also play a role in the decrease in metabolic activity, and histological assessments can be employed to evaluate this. Another limitation is the choice of anesthetic used in our CA model. Halothane has been shown to inhibit CYP2E1 in previous studies.(Tateishi, Watanabe et al. 1997) Since halothane has a very short half-life and that our CA model is conducted under a halothane washout period for 45 minutes prior to administration of CZN, we suggest that halothane is not contributing importantly to our observations. Another limitation is the duration of study as the half-life in the CA/hypothermia group exceeded the duration of sampling. Future studies are being developed to increase the duration of analysis in order to accurately capture changes in the half-life. Lastly, we conducted these experiments under moderate hypothermic conditions. Future experiments are being designed to identify the effect of mild hypothermia on CYP450-mediated metabolism, as this temperature range has been shown to be beneficial.

2.4 CONCLUSION

We report that hypothermia after CA alters the pharmacokinetic parameters of CZN, specifically showing reductions in the Cl_s , $t_{1/2}$, and K , and increases in the V_d . Our studies indicate that the

reductions in Cl_s are a result of temperature effects on the CYP2E1 enzyme, specifically, a decrease in the affinity of CZN for the CYP2E1 isoform. This is the first systematic investigation into the acute effect of hypothermic therapy on CYP2E1-mediated metabolism and the first mechanistic insight into hypothermia induced reductions in CZN clearance. Future studies are needed to assess the acute effects of hypothermia on other CYP450 isoforms as well as other enzymes which are involved in the metabolism of clinically relevant drugs. Although controlled clinical trials need to be employed in order to fully understand the effects of hypothermic therapy on the disposition of medications in the CA patient population, these data imply that clinically significant hypothermia-drug interactions are a likely factor to consider in the therapeutic course of these patients.

3.0 CHAPTER 3

Moderate Hypothermia Prevents Cardiac Arrest-Mediated CYP3A2 and CYP2E1 Suppression and Interleukin-6 Induction in the Rat

[Tortorici, MA, Kochanek PM, Xie W, and Poloyac SM. Moderate Hypothermia Prevents Cardiac Arrest-Mediated CYP3A2 and CYP2E1 Suppression and Interleukin-6 Induction. In preparation.

INTRODUCTION

The work outlined in Chapter 2, demonstrates that the application of moderate hypothermia (30°C) in a CA rat model, dramatically decreases the Cl_s of the CYP2E1 probe substrate, CZN (Tortorici, Kochanek et al. 2006). Furthermore, the mechanism behind the change in Cl_s was due to a decrease in the affinity of CYP2E1 for the substrate, CZN. These data from our study, along with data from other studies, demonstrate that during cooling there are dramatic changes in the disposition of medications metabolized by CYP450 enzymes in both animal and clinical models of injury (Koren, Barker et al. 1987; Iida, Nishi et al. 2001; Fukuoka, Aibiki et al. 2004). However, it is not known what happens to CYP450-mediated metabolism in the acute phase (immediately after re-warming, 5 hr after injury), the sub-acute phase (12-24 hrs after injury), the delayed phase (24-72 hrs after injury) or the chronic phase (72 hrs – 2 weeks after injury). As outlined in Chapter 1, one previous study investigated the effects of mild hypothermia on phenytoin pharmacokinetics in TBI patients and suggested that as a patient's core temperature is re-warmed to 37°C, the pharmacokinetic parameters of phenytoin normalize (Iida, Nishi et al. 2001). This may be due to the protective effects of therapeutic hypothermia on organs involved in drug metabolism. In order to fully understand the effects of hypothermia on drug metabolism it is also important to investigate the effects of hypothermia on drug disposition and metabolism once the patient's core temperature has been re-warmed, in order to optimize care.

Local or global inflammatory insults, such as CA, chronically regulate CYP450 expression and activity through the production of the acute phase response (APR), namely the release of pro-inflammatory cytokines, including interleukins 1 and 6 (IL-1 and IL-6) and tumor necrosis factor- α (TNF- α) (Carlson and Billings 1996; Khatsenko and Kikkawa 1997; Oppert, Gleiter et al. 1999; Roe, Poloyac et al. 2001; Frye, Schneider et al. 2002; Van Ess, Mattson et al. 2002; Haas, Kaufman et al. 2003). Furthermore, nitric oxide (NO) is an important biological mediator that is induced by multiple inflammatory stimuli and decreases the activity of CYP450 isoforms in experimental models of ischemia and endotoxemia (Carlson and Billings 1996; Khatsenko and Kikkawa 1997; Zhu, Deng et al. 2002). Hypothermia reduces the production of the acute phase response, specifically, pro-inflammatory cytokine production and NO after injury (Gundersen, Vaagenes et al. 2001; Han, Karabiyikoglu et al. 2003; Shima, Fujisawa et al. 2003; Truettner, Suzuki et al. 2005). Thus, we hypothesize that hypothermia treatment may provide protective effect on CYP450-metabolism after a global inflammatory insult, such as CA, in the acute and sub-acute phase after injury.

As mentioned in Chapter 1, CYP3A and CYP2E1 are both constitutively expressed in the rat and are regulated through transcriptional mechanisms (Rancid 2002). CYP3A2 is regulated through transcriptional mechanisms via hepatocyte nuclear factor-4 α (HNF-4 α) (Miyata, Nagata et al. 1995). CYP2E1 is also regulated through transcriptional mechanisms via hepatocyte nuclear factor-1 α (HNF-1 α) as well as post-translational events (Eliasson, Johansson et al. 1988; Eliasson, Mkrтчian et al. 1992; Roe, Poloyac et al. 2001; Cheng, Wang et al. 2003). Disease states which produce a potent APR, such as TBI and endotoxin administration, have been shown to decrease the expression and activity of both CYP3A2 and CYP2E1 in rat liver (Toler, Young et al. 1993; Roe, Poloyac et al. 2001; Cheng, Wang et al. 2003). However, the effects of CA on

CYP450 activity and expression as well as the potential protective effects of hypothermia applied after injury on this enzyme system have not been previously reported.

Based on this, we set out to examine the regulatory effects of CA and hypothermia on hepatic CYP3A2 and CYP2E1-mediated metabolism in the rat, in the acute and sub-acute phases after injury. The purpose of this study was to 1) examine the effects of CA and moderate hypothermia on the functional activity and mRNA expression of CYP3A2 and CYP2E1 in the rat liver at 5 and 24 hrs after injury, 2) examine if these changes are associate with induction of IL-6 and NO plasma concentrations as part of the acute phase inflammatory response in the rat after CA injury, and 3) determine if there are CA/hypothermia-mediated changes in HNF-4 α and HNF-1 α expression.

3.1 METHODS

3.1.1 Materials and Methods

Male Sprague-Dawley rats (body weight, 300-400 g) were purchased from Hilltop Laboratories (Scottsdale, PA). Chlorzoxazone (CZN), 6-hydroxychlorzoxazone (6-OH CZN), umbelliferone, and testosterone (TST) were purchased from Sigma Aldrich (St Louis, MO.) 6 β -hydroxytestosterone (6 β -OH TST) and 11 β -hydroxytestosterone (11 β -OH TST) were purchased from Steraloids (Newport,RI). Organic solvents were purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals were purchased from Sigma Aldrich unless otherwise specified.

3.1.2 Animal Cardiac Arrest and Hypothermia Protocol

The CA and Hypothermia protocol is outlined in detail in Chapter 2 and were similar in this study with modifications. Rats were allowed free access to food and water before and after surgery. The University of Pittsburgh Animal Care and Use Committee approved all studies. Sterile technique was used for all surgical procedures.

CA was conducted as previously described (Fink, Alexander et al. 2004; Katz, Young et al. 2004). Rats were anesthetized with 3% halothane via a nose cone, endotracheally intubated, and mechanically ventilated to maintain PaCO₂ between 35 to 45 mmHg. Halothane concentration was then reduced to between 0.5 to 1%. The right femoral artery was cannulated for measurement of mean arterial pressure and the femoral veins were cannulated bilaterally for drug administration and blood sampling. Neuromuscular blockade was induced with vecuronium 2 mg/kg (IV). A rectal probe was inserted for body temperature measurements. After preparative surgery, halothane was washed out with 100% O₂ for 3 minutes and room air for 2 minutes. Rats were randomized to receive either, anesthesia control normothermia (Control), CA with normothermia (CA Normothermia), or CA with hypothermia (CA Hypothermia), n=6. The use of a control hypothermia group was not warranted in this study due to the lack of clinical relevance. Animals received another vecuronium 1 mg/kg IV dose and CA was initiated by asphyxiation via disconnection of the mechanical ventilation in the animals in the CA treated groups. Asphyxial arrest was conducted for 8 minutes, with 5 minutes of asystole, confirmed by ECG trace as previously described (Fink, Alexander et al. 2004). Resuscitation was started by reconnecting the rat to the ventilator, administering epinephrine (0.005 mg/kg), IV and bicarbonate (1mEq/kg), IV and performing manual chest compressions for about one minute until restoration of spontaneous circulation (ROSC). Halothane was not

resumed after asphyxiation based on prior documentation of a burst-suppression pattern on EEG during early recovery (Fink, Alexander et al. 2004). Arterial blood gases and glucose measurements were taken prior to asphyxia and at the end of the re-warming phase. Acidosis was corrected via bicarbonate replacement to maintain pH at 7.4.

After ROSC, rats were either maintained at 37°C normothermia or body temperature was decreased to 30°C hypothermia over 30 minutes via surface cooling as depicted in **Figure 11**. Control rats core temperature was maintained at 37°C normothermia and received the same preparative surgery protocol without asphyxiation. Rats remained hypothermic over the entire 3 hour period after temperature stabilization. Intensive care management was performed in all animals and included as needed doses of vecuronium, bicarbonate, and butorphanol.

Rats were also randomized to one of two sacrifice time points, either 5 or 24 hrs after injury. Rats randomized to the 5 hr sacrifice group were sacrificed via decapitation at which time liver tissue was excised for analysis. Blood samples (0.3ml) were obtained from these rats via the left femoral venous cannula with a heparanized syringe at 60, 70, 90, 120, 180, 210, 240, 270, and 285 minutes after injury for IL-6 and NO plasma concentration analysis. Rats randomized to the 24 hr sacrifice group were gradually weaned from mechanical ventilation at the end of re-warming, extubated, and then returned to their cages. Rats were then sacrificed 24 hrs after injury via decapitation and liver tissue was excised for analysis.

3.1.3 Microsomal Preparation

Microsomes were prepared from liver tissue collected as previously described by Rockich *et al* (Rockich and Blouin 1999), via differential centrifugation in a Beckman L8-70 ultracentrifuge

(Beckman Instruments, Fullerton, CA). Total protein was determined by Lowry *et al.* (Lowry, Rosebrough *et al.* 1951).

3.1.4 CYP3A2 Enzyme Activity Analysis

Rat liver microsomal incubations contained 400 µg microsomal protein, 1 mM NADPH and 250µM of TST. These samples were incubated for 10 minutes at 37°C in a circulating water bath. The reaction was stopped by placing the samples on ice, followed by the addition of the internal standard, 11β-OH TST (50mcg/ml). 6β -OH TST was measured by a modified HPLC method described by Sonderfan *et al.* (Sonderfan, Arlotto *et al.* 1987). Briefly after the incubation and the addition of internal standard, 5 ml of dichloromethane was added to each sample. The organic phase was transferred and evaporated to dryness under a stream of nitrogen. Samples were then reconstituted with 200 µl of 50:50 methanol:ddH₂O. Mobile phase A consisted of filtered ddH₂O and Mobile phase B consisted of filtered methanol at a flow rate of 1.2 ml/min and 20 µl was injected onto the HPLC system. A gradient was established starting at 50:50 methanol:ddH₂O for 9 minutes, ramping over the next 3 minutes to 80:20 methanol:ddH₂O holding for 2 minutes, then ramping back to 50:50 methanol:ddH₂O over 2 minutes. 6β-OH TST, 11β-OH TST, and TST were separated using a Nova Pak C-18 reverse phase (5µm, 3.9 X 150mm) column (Waters, Millford, MA). The HPLC system consisted of a Waters 2695 with a 2487 UV detector, set at 254 nm. The ratio of 6β -OH TST to 11β-OH TST was used for quantitative analysis. The C.V. was less than 10% for this assay

3.1.5 CYP2E1 Enzyme Activity Analysis

CYP2E1 enzyme activity analysis was similar as outlined in Chapter 2, subsection 2.2.5 with modifications. Rat liver microsomal incubations contained 400 µg microsomal protein, 1 mM NADPH and 400µM of CZN. These samples were incubated for 20 minutes at 37°C in a circulating water bath. The reaction was stopped by adding 50 µl of 42.5% o-phosphoric acid, followed by the addition of the internal standard, umbelliferone (78µM). 6-OH CZN was measured by a modified HPLC method described by Rockich *et al.* (Rockich and Blouin 1999). Briefly, after the incubation and the addition of internal standard, 5 ml of diethyl ether was added to each sample. The organic phase was transferred and evaporated to dryness under a stream of nitrogen. Samples were then reconstituted with 200 µl of mobile phase A, consisting of 78% of a 0.25% acetic acid solution and 22% acetonitrile, and 20 µl was injected onto the HPLC system. Mobile phase B consisted of 95% acetonitrile and 5% of a 0.25% acetic acid solution and a gradient was established. CZN, umbelliferone, and 6-OH CZN were separated using a Nova Pak C-18 reverse phase (5µm, 3.9 X 150mm) column (Waters, Millford, MA). The HPLC system consisted of a Waters 2695 with a 2487 UV detector, set at 287 nm for CZN and 296 nm for 6-OH CZN. The ratio of CZN or 6-OH CZN to umbelliferone was used for quantitative analysis. The C.V. was less than 10% for this assay

3.1.6 Northern Blot Analysis

Total RNA was isolated from the liver of each rat using the TRIZOL method as previously described. Total RNA (20µg) was fractionated by electrophoresis on a 1.1% agarose gel, followed by a transfer onto a nylon membrane. Northern blots were hybridized with ³²P-labeled

oligonucleotides for CYP3A2 (5'-CGACTTGGAACCCATAGA-3'), CYP2E1 (5'-CTCCTCGTCATATCCATCTG-3'), as previously reported (Caron, Rioux et al. 2005), HNF-4 α (5'-TGACTACAGTGCTGCCTTGG-3'), HNF-1 α (5'-GAGAGGTGGTAGCAATTC-3'), and GAPDH (5'-ATCCCGCTAACATCAAATGG-3') as previously described (Podrig 2004). Semi-quantification was performed using a bio-imaging analyzer (Fluor-Chem).

3.1.7 Analysis of Interleukin-6 Plasma Concentrations

IL-6 concentrations were measured in the plasma of rats sacrificed 5 hours after injury using an enzyme linked immunosorbent assay (ELISA) kit specific for Rat IL-6 (R&D Systems, Minneapolis, MN). Briefly, plasma samples (50 μ L) was added to the assay diluent (50 μ L) and incubated for 2 hrs at room temperature. IL-6 conjugate (100 μ L) was added to each well and incubated for an additional 2 hrs, followed by the addition of 100 μ L of the substrate solution. The optical density was determined using a microplate reader set at 450 nm plus a correction at 540 to 570 nm.

3.1.8 Analysis of Nitrate and Nitrite Plasma Concentrations

NO₃ and NO₂ concentrations were measured in the plasma of rats sacrificed 5 hrs after injury. Plasma (100 μ l) was treated with 100 μ l of methanol and centrifuged at 8,000 rpm for 20 minutes. The supernatant of each sample was added to filter eppendorf tubes and then re-centrifuged at a speed of 14,000 rpm for 25 minutes. The supernatant of each sample was injected (10 μ l) using a Waters 717 autosampler (Waters, Milford, MA) and detected using a ENO-20 NO_x UV detector (EiCOM Corporation, San Diego). Total nitrate/nitrite concentrations were measured by

reducing the nitrate to nitrite with an in-line reduction column and detected at 540 nm using the Griess reaction.

3.1.9 Statistical Analysis

For comparisons of physiological variables and IL-6, NO₃ and NO₂ plasma concentrations analysis over time, a two-way analysis of variance (ANOVA) for repeated measures with a Bonferroni correction was used to compare Control, CA normothermia, and CA hypothermia groups. For comparisons of CYP2E1 and CYP3A2 activity and densitometric analysis of mRNA a one-way ANOVA with a Bonferroni correction was used to compared Control, CA normothermia, and CA hypothermia groups. We considered $p < 0.05$ to be statistically significant. Data are presented as mean \pm SD. All analyses were conducted using the software program Prism (GraphPad Software, San Diego, CA).

3.2 RESULTS

3.2.1 Time Course of Hypothermia in Cardiac Arrest Treated Rats

As depicted in **Figure 11**, Control and CA Normothermia rats were maintained at 37°C and CA Hypothermia rats were cooled to 30°C over 30 minutes and maintained for 3 hours followed by 1 hour of re-warming.

3.2.2 Physiologic Variables Cardiac Arrest and Hypothermia Treated Rats

Physiological parameters (MABP, blood pH, PaO₂, PaCO₂, and glucose) were measured (Table 6) in Control, CA Normothermia, and CA Hypothermia groups. The pre-CA/hypothermia measurements were drawn after preparative surgery and immediately prior to the initiation of CA. The post-CA/hypothermia measurements were drawn in the acute-phase after injury and at the analogous time points in other experimental groups. No differences in physiologic parameters measured between all 3 groups before CA. However, post-CA/hypothermia parameters demonstrate that CA Normothermia and CA Hypothermia groups have an anticipated significant increase in PaO₂ and decrease in PaCO₂, when compared to pre-CA/hypothermia parameters (**p<0.01) (Fink, Alexander et al. 2004). Furthermore, post-CA/hypothermia values of PaO₂ and PaCO₂ in CA Normothermia and CA Hypothermia groups were significantly different from the Control group (**p<0.001). There were no other differences between groups.

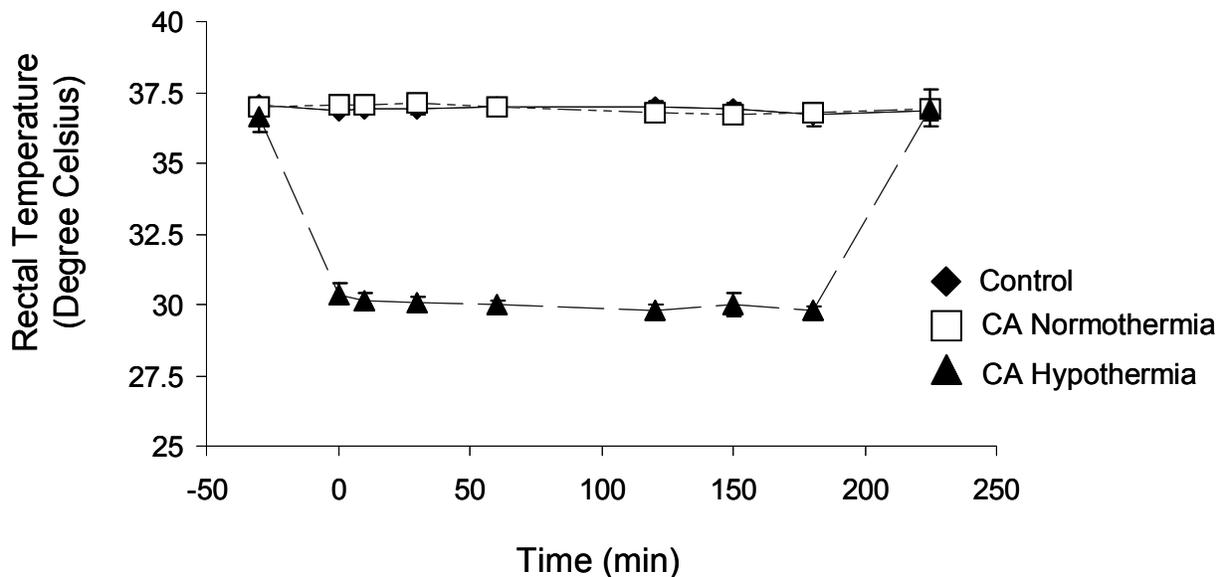


Figure 11: Time-course of rectal temperature beginning 15 minutes after asphyxial arrest

Control and CA Normothermia rats were maintained at 37°C, and CA Hypothermia rats were maintained at 30°C for 3 hrs followed by 1 hr re-warming

Table 6: Physiological variables in rats

	Experimental Group	Pre-CA/hypothermia	Post-CA/hypothermia
MABP (mmHg)	Control	98.58 ± 8.55	102.8 ± 13.7
	CA Normothermia	105.0 ± 13.7	122.5 ± 20.1
	CA Hypothermia	105.8 ± 8.49	111.7 ± 34.5
pH	Control	7.37 ± 0.022	7.36 ± 0.077
	CA Normothermia	7.38 ± 0.032	7.39 ± 0.047
	CA Hypothermia	7.38 ± 0.19	7.37 ± 0.085
PaCO ₂ (mmHg)	Control	39.93 ± 3.84	39.85 ± 7.83
	CA Normothermia	38.16 ± 2.55	25.22 ± 6.89 ^{***,†††}
	CA Hypothermia	38.50 ± 3.50	21.81 ± 5.71 ^{***,†††}
PaO ₂ (mmHg)	Control	244.4 ± 15.7	227.38 ± 58.7
	CA Normothermia	236.3 ± 25.8	417.54 ± 76.2 ^{***,†††}
	CA Hypothermia	234.4 ± 38.1	463.54 ± 67.4 ^{***,†††}
Glucose (mg/dL)	Control	114.5 ± 20.2	121.9 ± 27.1
	CA Normothermia	120.6 ± 32.5	108.5 ± 24.4
	CA Hypothermia	116.3 ± 16.9	136.5 ± 28.2

Abbreviations: MABP, mean arterial blood pressure; PaCO₂, pressure of carbon dioxide; PaO₂, pressure of oxygen

3.2.3 Effect of Cardiac Arrest and Hypothermia on 6β-hydroxytestosterone Formation Rate in Rat Liver 5 and 24 Hours After Injury

Figure 12, Panel A depicts the formation rate of 6β-OH TST in liver microsomes from the 3 treatments groups, Control, CA Normothermia, and CA Hypothermia (n=6) in rats sacrificed in the acute phase, 5 hrs after injury. 6β-OH TST formation rate showed no difference between Control (2388 ± 513 pmol/mg/min), CA Normothermia (2374 ± 299 pmol/mg/min), and CA Hypothermia (2521 ± 689 pmol/mg/min) groups. **Figure 12, Panel B** depicts the formation rate of 6β-OH TST in liver microsomes from the 3 treatment groups, (n=6) in rats sacrificed in the sub-acute phase, 24 hrs after injury. 6β-OH TST formation rate was significantly decreased in the CA Normothermia rats compared to Control rats (1484 ± 942.9 pmol/mg/min vs. 3174 ±

965.4 pmol/mg/min; * $p < 0.05$, respectively). 6 β -OH TST formation rate (4345 ± 1198 pmol/mg/min) was not significantly different between CA Hypothermia and Control rats, however a significant difference was observed between CA Hypothermia and CA Normothermia rats; ‡ $p < 0.05$.

3.2.4 Effect of Cardiac Arrest and Hypothermia on *CYP3A2* mRNA Expression in Rat Liver 5 and 24 Hours After Injury

Rats sacrificed in the acute phase, 5 hrs after injury in all three groups (Control, CA Normothermia, and CA Hypothermia) demonstrated no difference in *CYP3A2* mRNA expression as shown in **Figure 13, Panel A**. There was no difference in band density between groups (**Figure 13, Panel B**; $n=6$). However, significant differences were observed in the sub-acute phase, 24 hrs after injury, demonstrating a decrease in CA Normothermia group in *CYP3A2* mRNA expression compared to both Control and CA Hypothermia as depicted in **Figure 13, Panel C**. **Figure 13, Panel D** ($n=6$), demonstrates a significant difference in the band density of *CYP3A2* mRNA expression in the CA Normothermia rats (* $p < 0.05$), compared to Control rats. Furthermore, no difference was observed in the sub-acute phase, 24 hrs after injury in the CA Hypothermia rats compared to Control rats.

3.2.5 Effect of Cardiac Arrest and Hypothermia on 6-hydroxychlorzoxazone Formation Rate in Rat Liver 5 and 24 Hours After Injury

Figure 14, Panel A depicts the formation rate of 6-OH CZN in liver microsomes from the 3 treatments groups, Control, CA Normothermia, and CA Hypothermia (n=6) in rats sacrificed acute phase, 5 hrs after injury. No significant difference in 6-OH CZN formation rate was observed between Control (1.59 ± 0.34 nmol/mg/min), CA Normothermia (1.62 ± 0.31 nmol/mg/min), and CA Hypothermia (1.73 ± 0.36 nmol/mg/min) groups. **Figure 14, Panel B** depicts the formation rate of 6-OH CZN in liver microsomes from the 3 treatment groups, (n=6) in rats sacrificed in the sub-acute phase, 24 hrs after injury. CA Normothermia showed a significant decrease in 6-OH CZN formation rate compared to Control (0.472 ± 0.11 nmol/mg/min versus 0.931 ± 0.144 nmol/mg/min; $p < 0.05$, respectively). 6-OH CZN formation rate was not significantly different between CA Hypothermia and Control rats, however a significant difference was observed between CA Hypothermia and CA Normothermia rats; $\ddagger p < 0.05$.

3.2.6 Effect of Cardiac Arrest and Hypothermia on *CYP2E1* mRNA Expression in Rat Liver at 5 and 24 Hours After Injury

Rats sacrificed in the acute phase, 5 hrs after injury in all three groups (Control, CA Normothermia, and CA Hypothermia) demonstrated no difference in *CYP2E1* mRNA expression as shown in **Figure 15, Panel A & B** (n=6). A trend towards a decrease in *CYP2E1* mRNA expression was observed in the sub-acute phase, 24 hrs after injury between CA Normothermia as compared to the Control and CA Hypothermia; **Figure 15, Panel C**. However, these differences in density were not statistically significant as depicted in **Figure 15, Panel D** (n=6).

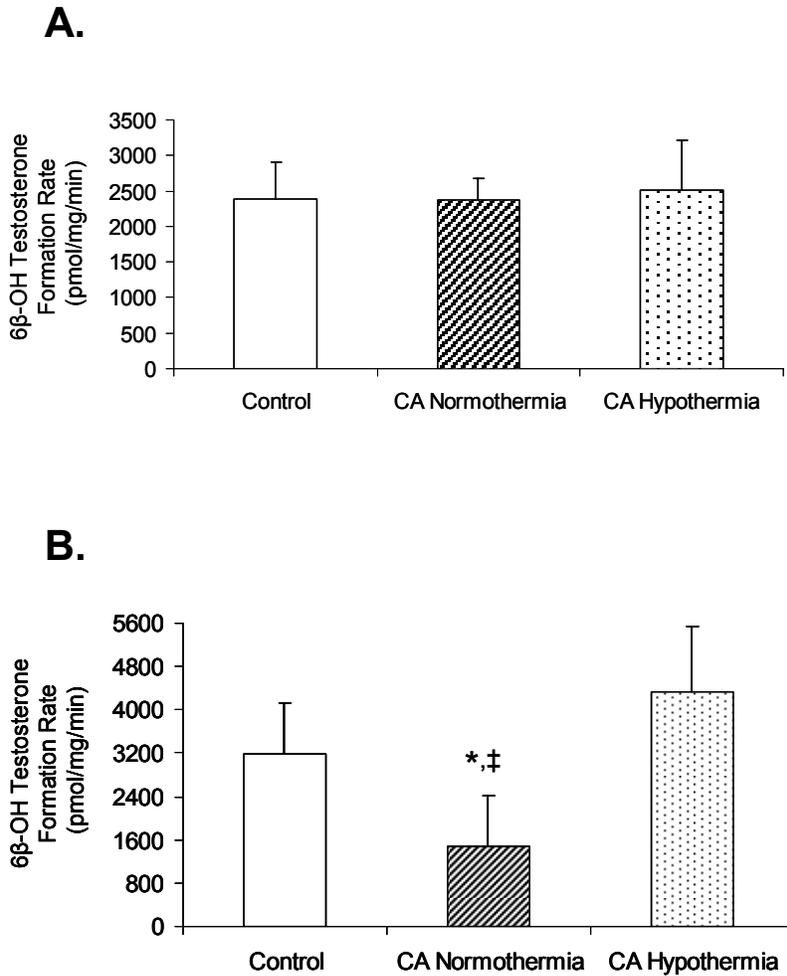


Figure 12: CYP3A2 activity in rat liver microsomes

Panel A depicts 6β-hydroxytestosterone formation rate in rats sacrificed 5 hrs after injury. Data demonstrates no differences were observed between Control (2388 ± 513 pmol/mg/min), CA Normothermia (2374 ± 299 pmol/mg/min, and CA Hypothermia (2521 ± 689 pmol/mg/min). **Panel B** depicts 6β-hydroxytestosterone formation rate in rats sacrificed 24 hrs after injury. Data demonstrates that activity in CA Normothermia (1484 ± 942 pmol/mg/min) was significantly decreased compared to both Control (3174 ± 965.4; *p<0.05) and CA Hypothermia (4345 ± 1198; ‡p<0.05) rats. All data are presented as mean ± SD

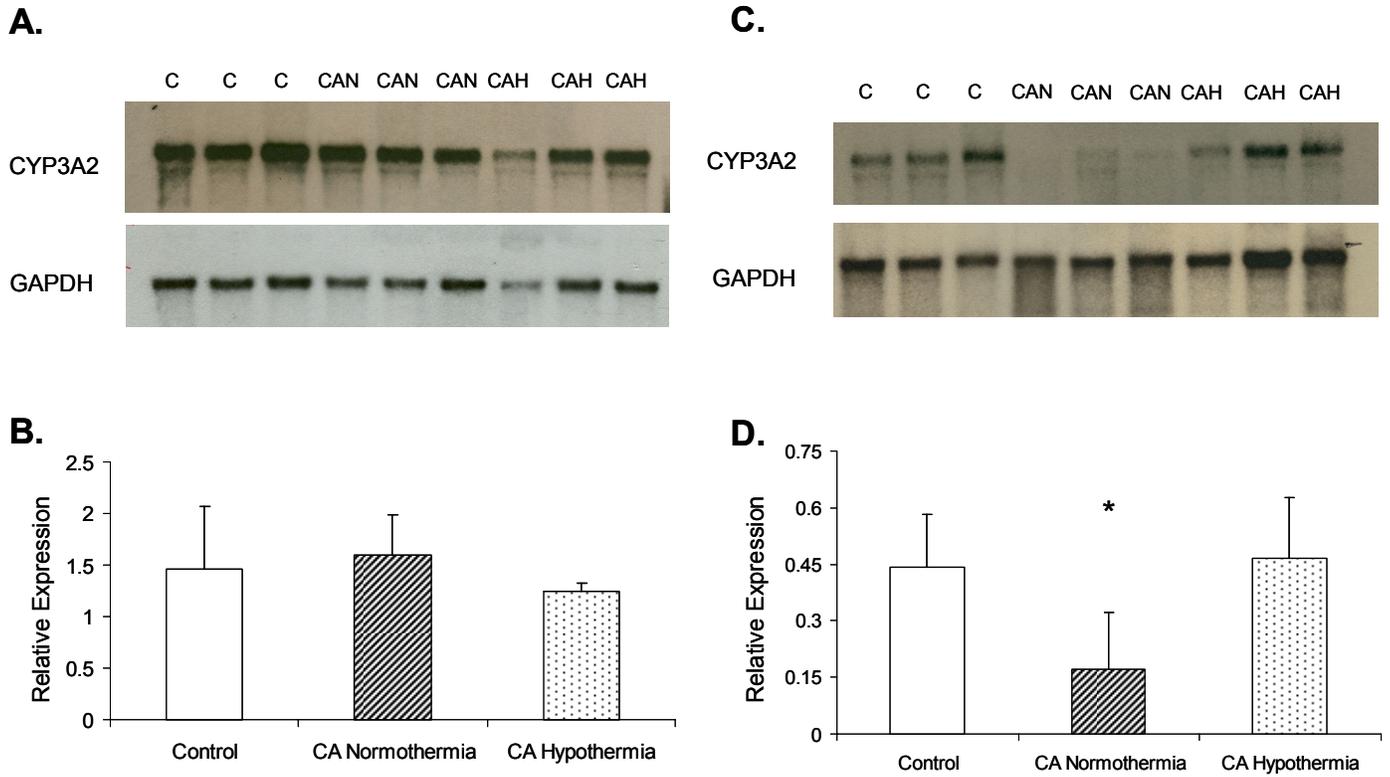
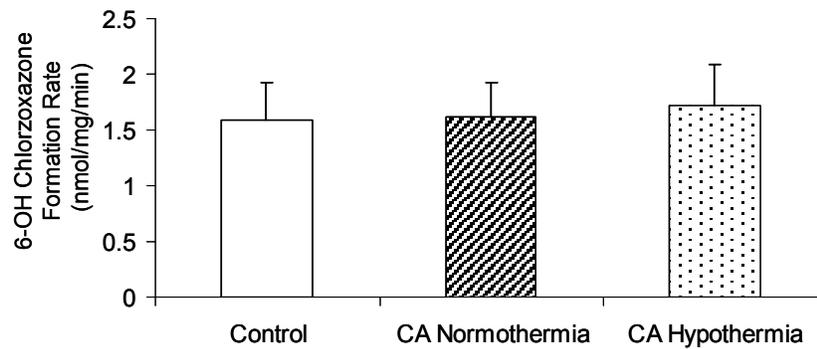


Figure 13: *CYP3A2* mRNA expression in rat liver

Panel A depicts *CYP3A2* mRNA expression 5 hrs after injury in all 3 groups. Data demonstrates no difference between groups, as shown upon densitometric analysis in Panel B. Panel C depicts *CYP3A2* mRNA expression 24 hrs after injury in all 3 groups. Data demonstrates a significant decrease *CYP3A2* relative hepatic expression in the CA Normothermia rats compared to Control and CA Hypothermia, as shown upon densitometric analysis in Panel D; $p < 0.05$. All data are presented as mean \pm SD

A.



B.

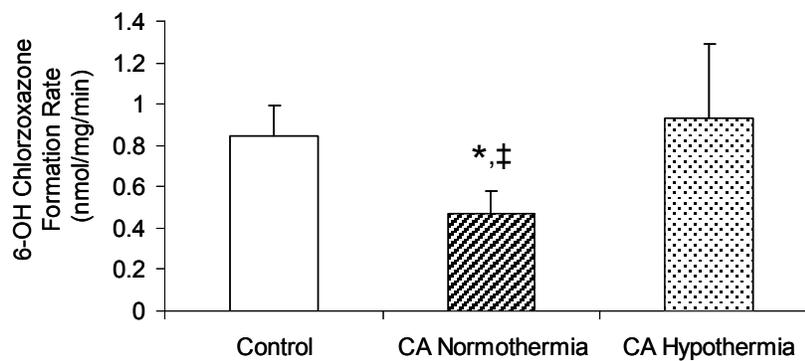


Figure 14: CYP2E1 activity in rat liver microsomes.

Panel A depicts 6-hydroxychlorzoxazone formation rate in rats sacrificed 5 hrs after injury. Data demonstrates no differences were observed between Control (1.59 ± 0.34 nmol/mg/min), CA Normothermia (1.62 ± 0.31 nmol/mg/min, and CA Hypothermia (1.73 ± 0.36 nmol/mg/min). Panel B depicts 6-hydroxychlorzoxazone formation rate in rats sacrificed 24 hrs after injury. Data demonstrates that activity in CA Normothermia (0.472 ± 0.11 nmol/mg/min) was significantly decreased compared to both Control (0.931 ± 0.14 nmol/mg/min; * $p < 0.05$) and CA Hypothermia (0.931 ± 0.36 nmol/mg/min; † $p < 0.05$) rats. All data are presented as mean \pm SD

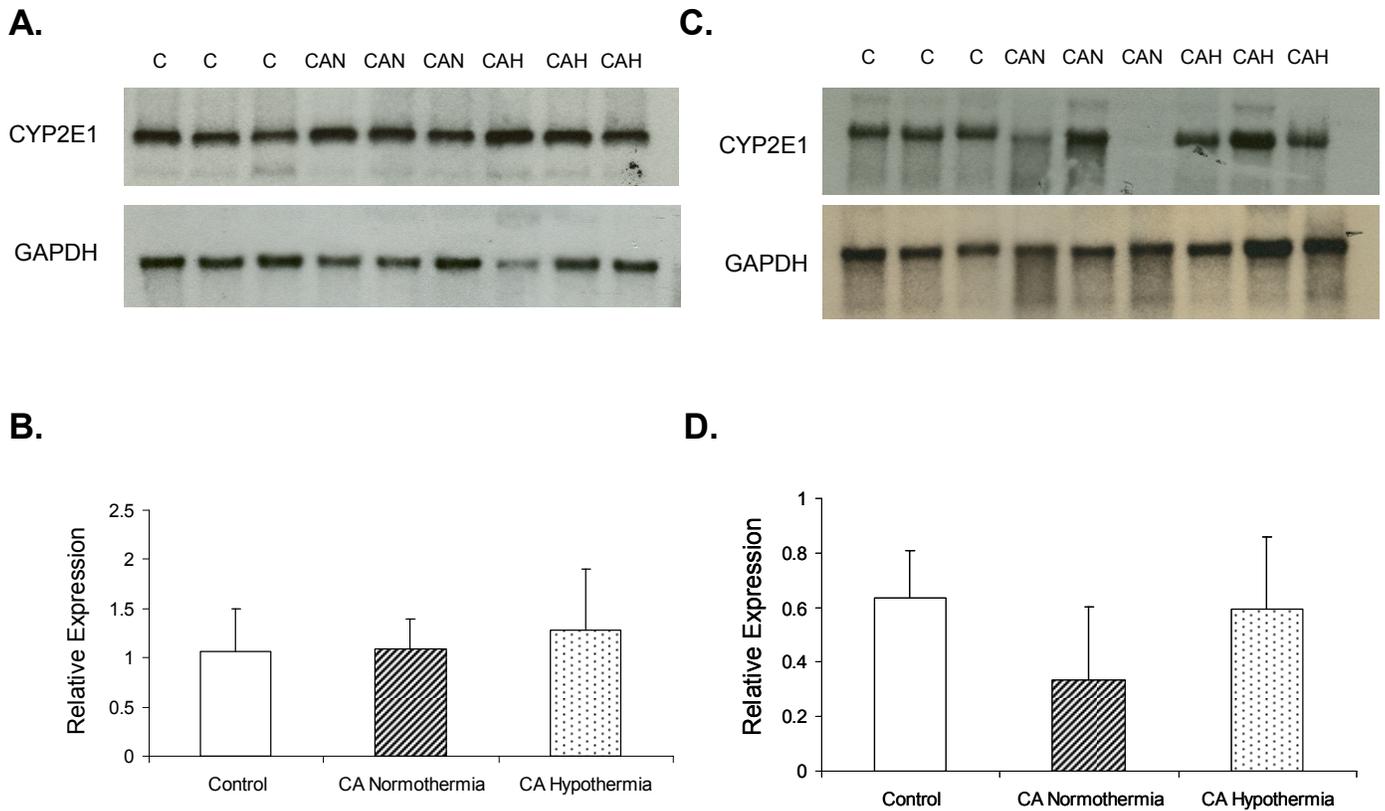


Figure 15: *CYP2E1* mRNA expression in rat liver

Panel A depicts *CYP2E1* mRNA expression 5 hrs after injury in all 3 groups. Data demonstrates no difference between groups, as shown upon densitometric analysis in Panel B. Panel C depicts *CYP2E1* mRNA expression 24 hrs after injury in all 3 groups. Data demonstrates a trend towards a decrease *CYP2E1* relative hepatic expression in the CA Normothermia rats compared to Control and CA Hypothermia, but did not reach significance as shown upon densitometric analysis in Panel D. All data are presented as mean \pm SD

3.2.7 Effect of Cardiac Arrest and Hypothermia on IL-6 Plasma Concentrations

Figure 15 depicts the time-course of plasma concentrations of IL-6 in 3 groups, Control, CA normothermia, and CA hypothermia; n=6 after injury. CA normothermia produced significant increases in IL-6 concentrations compared to control 60, 70, and 90 minutes after injury; ***p<0.001. After 90 minutes post-injury, IL-6 plasma concentrations returned to levels comparable to the Control group and were maintained throughout the rest of the study.

Interestingly, when hypothermia was applied in the CA model, IL-6 levels demonstrated no difference compared to Control animals at all time points.

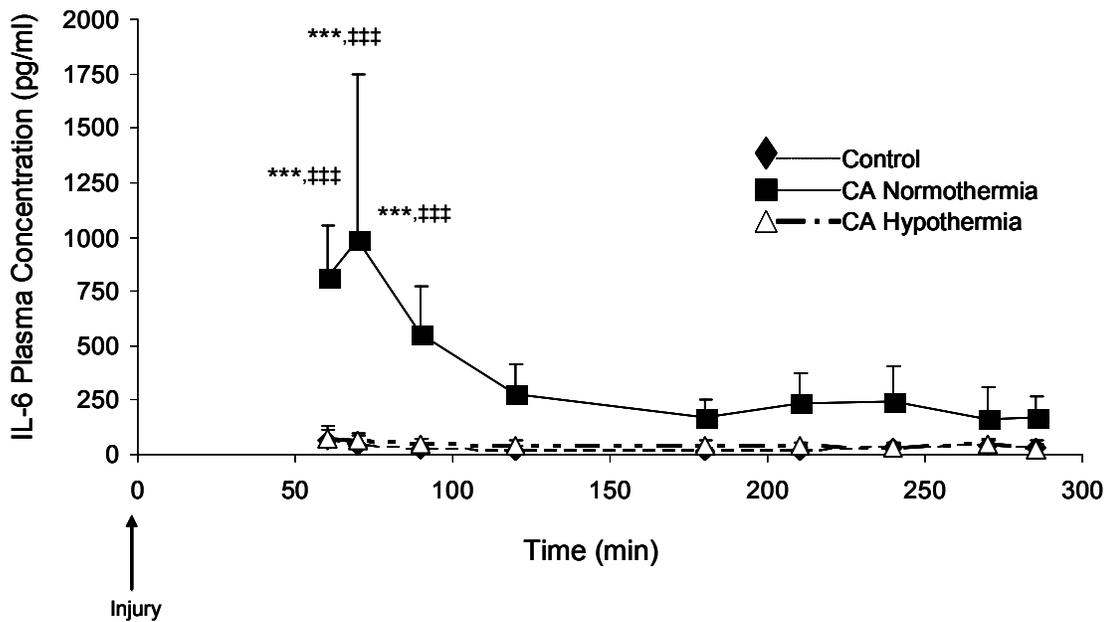


Figure 16: Plasma interleukin-6 (IL-6) plasma concentrations

Data demonstrates that in CA Normothermia there were significant increases in IL-6 plasma concentrations at 60, 70, and 90 minutes after injury, compared to Control (***) and CA Hypothermia (###). All data are presented as mean \pm SD.

3.2.8 Effect of Cardiac Arrest and Hypothermia on NO₂ and NO₃ Concentrations in Rat Plasma

Figure 17A and **17B** depicts a time course of NO₂ and NO₃ concentrations in rat plasma in all 3 groups; Control, CA normothermia, and CA hypothermia, n=6. A trend towards an increase was observed in the CA Normothermia rats; however this did not reach significance. No differences in NO₂ were observed between groups at all time points.

3.2.9 Effect of Cardiac Arrest and Hypothermia on *HNF-4α* Expression in Rat Liver 24 Hours After Injury

Significant differences were observed in the sub-acute phase, 24 hrs after injury, demonstrating a significant decrease in CA Normothermia group in *HNF-4α* mRNA expression compared to both Control and CA Hypothermia as depicted in **Figure 18, Panel A**. **Figure 18, Panel B** (n=6), demonstrates a significant difference in the band density of *HNF-4α* mRNA expression in the CA Normothermia rats, compared to Control (**p<0.001) and CA Hypothermia (‡p<0.05) rats. Furthermore, no difference was observed in the sub-acute phase, 24 hrs after injury in the CA Hypothermia rats compared to Control rats.

3.2.10 Effect of Cardiac Arrest and Hypothermia on *HNF-1α* Expression in Rat Liver 24 Hours After Injury

There were no differences observed in the sub-acute phase, 24 hrs after injury, in *HNF-1α* mRNA expression in all three groups as depicted in **Figure 19, Panel A**. **Figure 19, Panel B** (n=6), demonstrates no difference in the band density of *HNF-1α* mRNA expression in all three groups.

3.3 DISCUSSION

Our results demonstrate four major findings. First, CA produces a significant decrease in the hepatic activity of CYP3A2 and CYP2E1 in rats, in the sub-acute phase, 24 hrs after injury compared to Control rats. Second, there is an increase in IL-6 plasma concentrations early after CA. IL-6 is known to decrease *CYP3A2* and *CYP2E1* expression. Third, CA produced a decrease in the expression of the constitutive transcription factor, *HNF-4 α* , 24 hrs after injury, but did not change the expression of *HNF-1 α* . Fourth, moderate hypothermia applied to CA rats, prevented both the transcriptional down-regulation of CYP3A2 and CYP2E1-metabolism and blocked the early increase in IL-6 seen after CA Normothermia. These findings suggest that by blocking the inflammatory response to tissue injuries after CA, hypothermia may favorably alter the natural course of disease and normalize CYP450-mediated drug metabolism.

CA produces a decrease in hepatic activity and expression of CYP3A2 and CYP2E1 in Rat Liver in the sub-acute phase, 24 hrs after injury. CA produces a significant decrease in the activity of both CYP3A2 and CYP2E1 in the sub-acute phase, 24 hours after injury. Furthermore, we demonstrated that this decrease in activity was paralleled by a decrease in *CYP3A2* mRNA, whereas, significant changes in *CYP2E1* mRNA were not observed. This is the first investigation which has specifically identified the

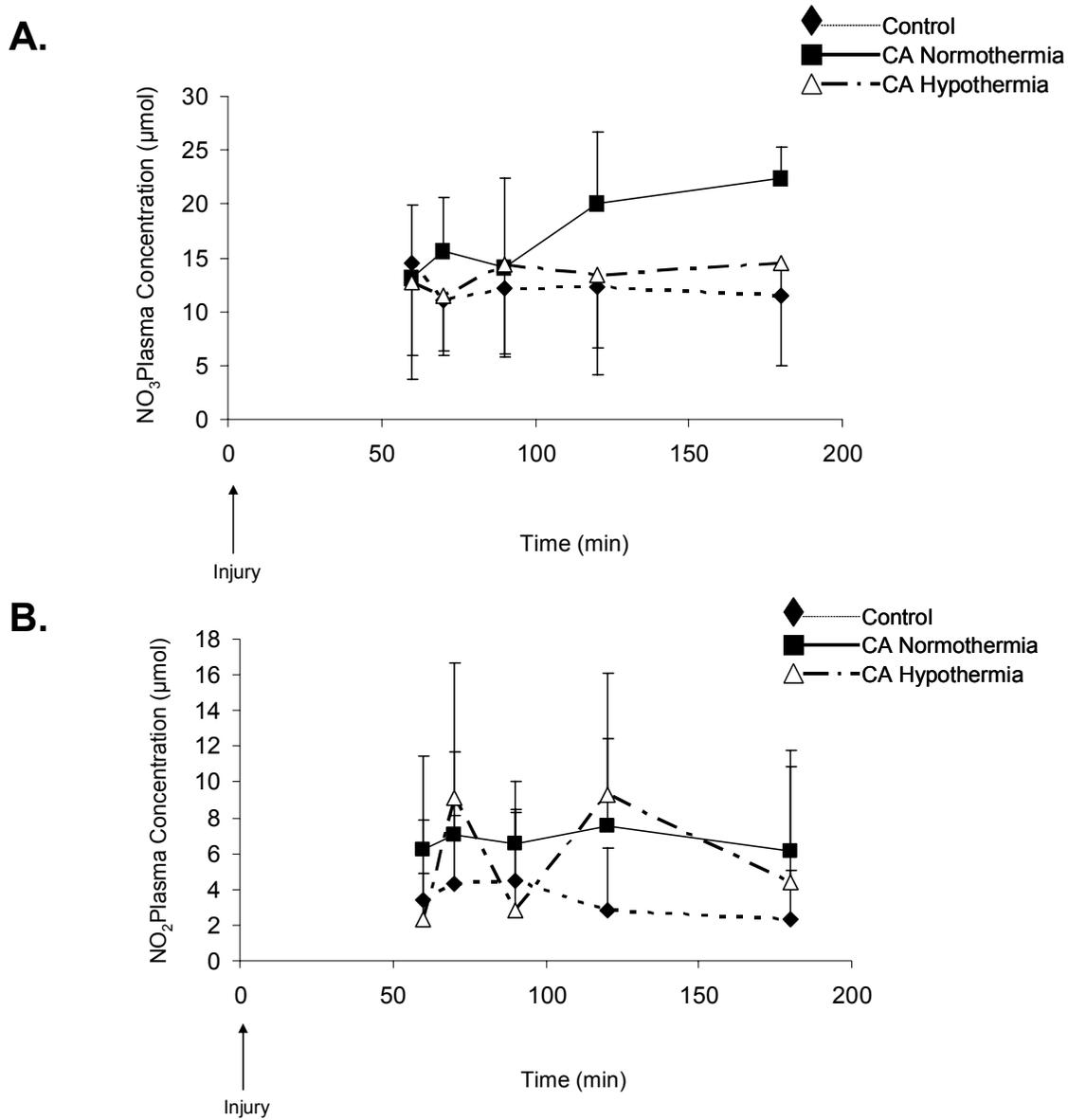


Figure 17: Nitrate and nitrite plasma concentrations

Panel A: Plasma nitrate (NO₃) concentrations in all three groups, Control, CA Normothermia, and CA Hypothermia after injury. A trend towards an increase was observed in the CA Normothermia rats; however this did not reach significance. **Panel B:** Plasma nitrite (NO₂) concentrations in all three groups, Control, CA Normothermia, and CA Hypothermia after injury. No differences were observed between groups at all time-points. All data are presented as mean ± SD.

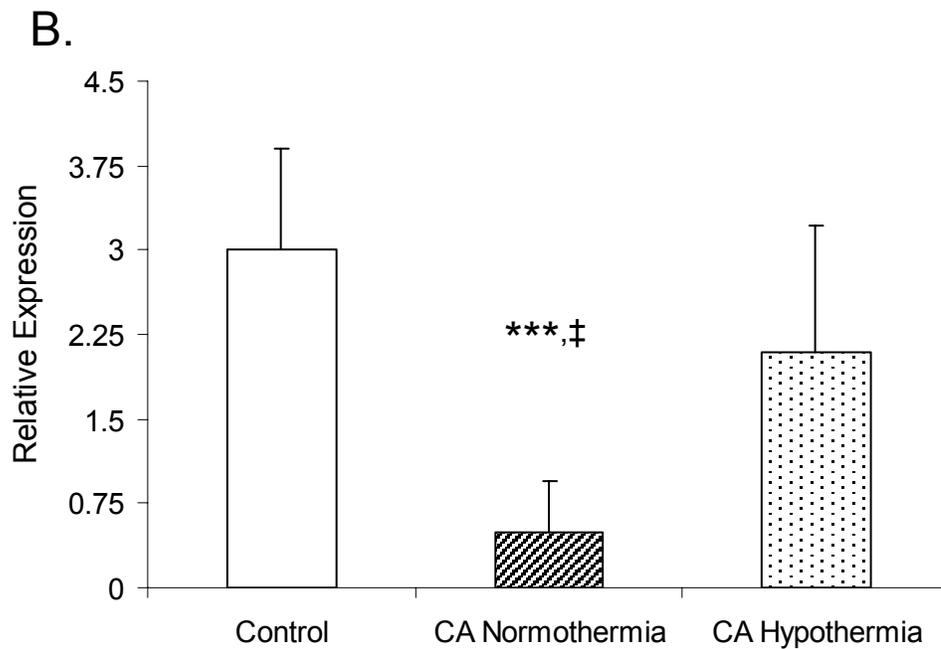
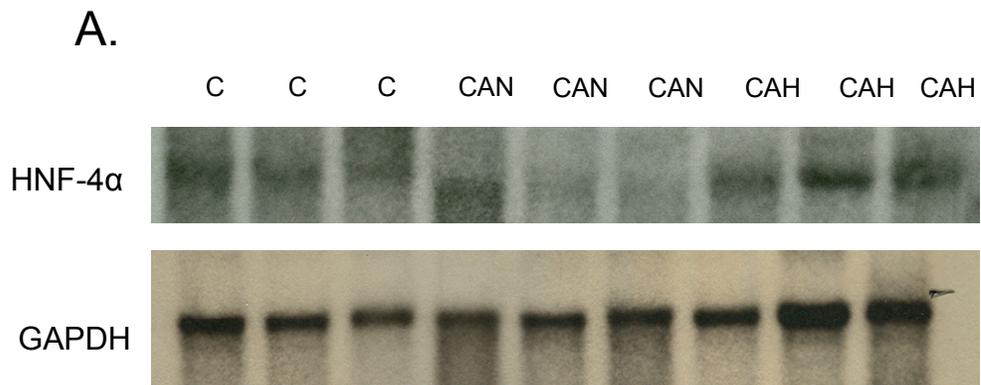


Figure 18: *HNF-4α* expression in rat liver

Data shows a significant decrease in CA Normothermia rats 24 hours after injury in *HNF-4α* expression compared to Control ($p < 0.001$) and CA Hypothermia ($p < 0.05$) after densitometric analysis. No difference was observed between Control and CA Hypothermia rats.

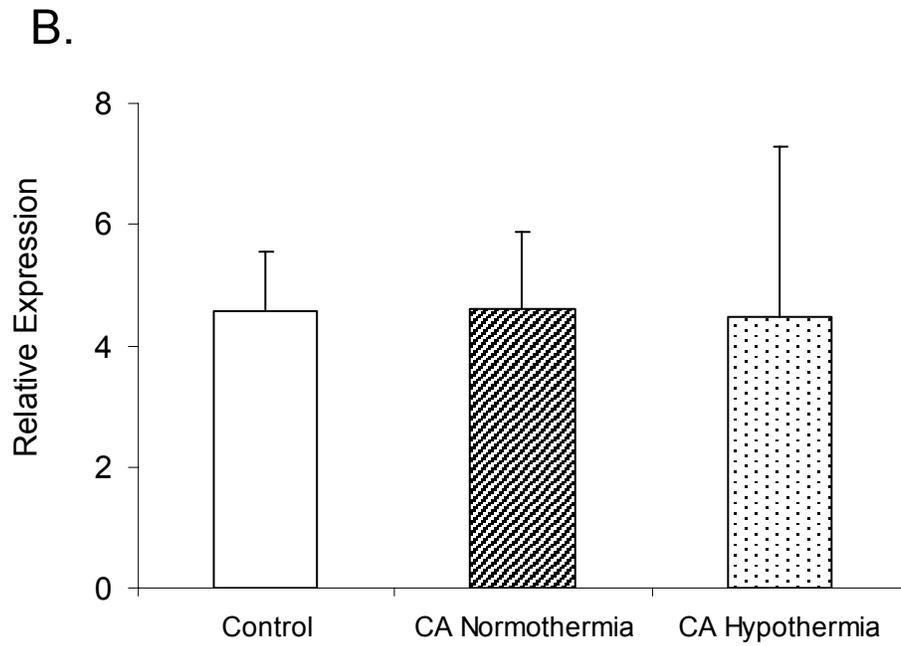
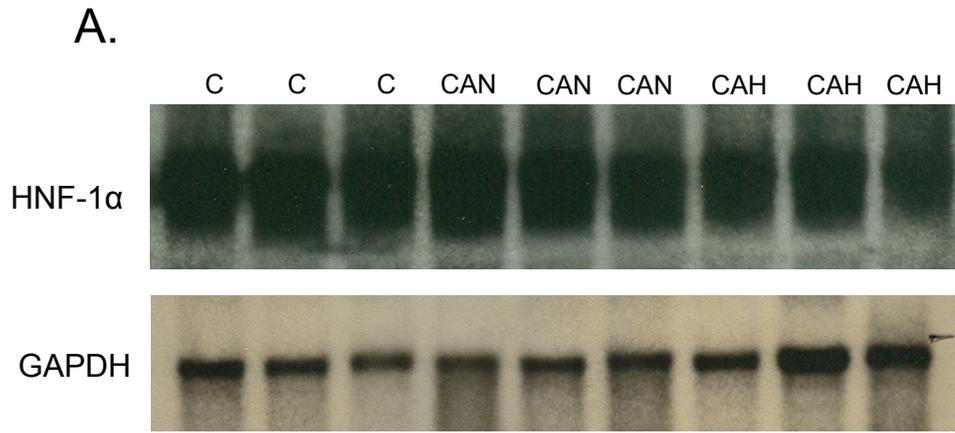


Figure 19: *HNF-1 α* expression in rat liver

No difference was observed between all 3 groups in *HNF-1 α* expression, 24 hours after injury.

effects of CA injury on CYP450-mediated metabolism; however studies have shown that in other models of ischemic injury, such as TBI, decreases in the expression and activity of multiple CYP450 isoforms are evident (Toler, Young et al. 1993; Boucher and Hanes 1998; Kalsotra, Turman et al. 2003). Toler et al, showed that in TBI rats, hepatic CYP2C11 and CYP3A mRNA expression were decreased ~50% of control, 24-hours after injury. Interestingly, they demonstrated no difference in hepatic activity of either isoform, 24 or 48 hours after injury (Toler et al., 2003). Kalsotra et al showed that after TBI, rats demonstrated no difference in hepatic CYP3A protein and activity 24 hrs after injury, but showed a dramatic increase in both hepatic activity and protein content 2 weeks after injury (Kalsotra, Turman et al. 2003). Poloyac et al demonstrated that there was no change in the hepatic activity of CYP2E1, 24 hrs after TBI in rats (Poloyac, Perez et al. 2001). Unlike TBI, CA is a global ischemic event to the entire organism and therefore, may elicit a greater systemic acute phase response, potentially resulting in greater effects on hepatic CYP450-mediated metabolism (Oppert, Gleiter et al. 1999; Wilcockson, Campbell et al. 2002). Compared to Control, our CA rat model demonstrated a reduction of ~55% in CYP2E1 activity and 47% in CYP3A2 activity. Collectively, these studies suggest that ischemic and traumatic injuries, such as CA and TBI, down-regulate CYP450 activity and expression in the sub-acute phase, 24 hrs after injury.

CA produces increases in IL-6 plasma concentrations after injury. CA produces a potent acute phase response (Oppert, Gleiter et al. 1999). We found that CA produced a ~10-fold increase in IL-6 plasma concentrations at 60, 70, and 90 minutes after injury compared to Control. IL-6 mediates the majority of the hepatic alterations of the acute phase response and is most potent inhibitor of CYP450s of all the pro-inflammatory cytokines (Hakkola, Hu et al. 2003). Other models of ischemic injury have shown that increased plasma concentrations of IL-

6 after injury is associated with a decreased activity of CYP450 isoforms (Toler, Young et al. 1993). Supporting cause and effect, the addition of IL-6 to hepatocytes decreases the mRNA expression of both *CYP2E1* and *CYP3A2* (Abdel-Razzak, Loyer et al. 1993). The mechanism of the decreases in CYP450 activity have been shown to be due to a cytokine-mediated down-regulation in the transcription of CYP450 isoforms (Abdel-Razzak, Loyer et al. 1993; Toler, Young et al. 1993; Hakkola, Hu et al. 2003). In addition, our data demonstrates that CA did not produce a significant increase in both NO₂ and NO₃ early after injury. It has been well established that NO production increases after injury in models of ischemia and endotoxemia, and has been shown to suppress CYP450 functional activity (Carlson and Billings 1996; Khatsenko and Kikkawa 1997). Interestingly, these studies show that peak production of NO is ~ 24-48 hrs after ischemia or LPS injection. It is possible that our results do not show a difference because we analyzed plasma at the early time-points (1-3 hrs) after CA. However, this does not rule out the role of NO on the decrease in CYP activity we observed. Collectively, our results suggest that cytokine alterations after a global ischemic insult may produce a reduction in the functional regulation of CYP3A2.

CA decreases the expression of the constitutive transcription factor, HNF-4. Our data demonstrates a decrease in the expression of *HNF-4α*, 24 hrs after CA compared to Control. However, we did not observe any change in the expression of *HNF-1α*, 24 hrs after CA compared to Control. *HNF-4α* and *HNF-1α* have both been shown to constitutively regulate *CYP3A2* and *CYP2E1*, respectively (Miyata, Nagata et al. 1995; Roe, Poloyac et al. 2001). Mizuguchi *et al* determined that the addition of IL-6 to rat hepatocytes decreased the expression of *HNF-4*, however did not affect the expression of *HNF-1α* (Mizuguchi, Mitaka et al. 1998). Other studies have supported this finding, demonstrating that IL-6 is not involved in the

reduction of HNF-1 α binding (Green, Beier et al. 1996; Trauner, Arrese et al. 1998). Therefore, the reduction in the activity of CYP2E1 24 hrs after injury cannot be explained by changes in transcriptional regulation. Although other cytokines, such as IL-1 β and TNF- α , may be important inflammatory mediators after CA, our data lends evidence to the finding that the production in IL-6 after injury is followed by a decrease in the expression of HNF-4 α , 24 hrs after injury. Collectively, these results propose a potential mechanism in the down-regulation of CYP3A2 after CA.

Moderate hypothermia provides a protective effect on CYP3A2 and CYP2E1 activity and expression. When rats were treated with moderate hypothermia after CA, we observed no difference in the activity and expression of both CYP3A2 and CYP2E1 compared to Control. Few studies have been conducted to evaluate the effect of hypothermia on CYP regulation. However, a clinical study that provides insight into these effects was performed by Iida, Nishi et al. (2001). This study investigated the both the acute and chronic effects of mild hypothermia in TBI patients on phenytoin pharmacokinetics. Phenytoin is metabolized primarily through the CYP2C9 and CYP2C19 isoforms. Acutely, hypothermia decreases the Cl_s of phenytoin; however, after the patients core temperature was returned to normothermic temperatures, the Cl_s of phenytoin was returned to normal. Our laboratory has demonstrated that during cooling, moderate hypothermia decreases the Cl_s ~2-fold, of the CYP2E1 probe substrate, CZN (Tortorici, Kochanek et al. 2006). Hypothermia has been shown to attenuate the release of certain pro-inflammatory cytokines after TBI and hemorrhagic shock in rats, which may play a role for the protection incurred by hypothermia in our model (Gundersen, Vaagenes et al. 2001). Our data supports these studies, as we showed that the application of moderate hypothermia in a CA rat model prevented the increase in IL-6 concentrations we observed in CA Normothermia

rats. Collectively, these data show that hypothermia applied to ischemic injury models, normalizes drug metabolism in the sub-acute phase after injury. This suggests that after various insults such as CA, hypothermia may inhibit drug metabolism during cooling, but that the benefits of moderate hypothermia on tissue injury and inflammation may favorably alter the pathogenic processes of the disease, thereby ultimately normalizing drug metabolism.

Limitations. There are several limitations of our study. First, we investigated the effects of CA and hypothermia on CYP3A2 and CYP2E1 metabolism at two time-points after injury, 5 and 24- hours. Animal models of inflammation have demonstrated changes in CYP450 expression due to the acute phase response at multiple time-points after LPS administration (Roe, Poloyac et al. 2001; Cheng, Wang et al. 2003). Although we chose relevant time-points, a complete time-course of alterations in CYP450 activity and expression after CA injury and hypothermia is necessary for a full understanding of these alterations. This could have substantial importance to clinical application since cooling is induced for 12-24 hrs (Bernard, Gray et al. 2002). Second, we investigated one pro-inflammatory cytokine, (IL-6), due to the fact that it is thought to be the most important mediator of hepatic changes of the acute phase response (Ramadori and Christ 1999). Other cytokines, such as IL-1 β and TNF- α have also been shown to contribute importantly to the down-regulation of CYP450 enzymes (Hakkola, Hu et al. 2003). In addition, we showed no difference in NO plasma concentrations in CA rats at early time points after injury. As mentioned above, NO production peaks between 24-48 hrs after ischemic injury making it possible that we did not characterize a change which may contribute importantly to the decrease in CYP3A2 and CYP2E1 activity (Khatsenko and Kikkawa 1997). Further investigation of multiple cytokines and NO concentrations at multiple time-points after CA will provide a greater understanding of the multiple factors resulting from this global ischemic injury.

Lastly, although our data are consistent with results other models of inflammation on CYP450 alterations, future studies are needed to fully elucidate the effects of CA on CYP450 metabolism and the protective effects of therapeutic hypothermia.

3.4 CONCLUSION

This study shows that after CA, a decrease in both the hepatic activity and expression of CYP3A2 and CYP2E1 isoforms in the sub-acute phase, 24 hrs after CA as compared to Control. A potential mechanism is via an IL-6-mediated down-regulation of the expression of *HNF-4 α* resulting in a decrease in CYP3A2 transcription. In addition, no difference was observed in the early production after injury of NO₂ and NO₃ in CA-rats compared to Control. Furthermore, the use of moderate therapeutic hypothermia attenuated the CA-mediated decrease in CYP3A2 and CYP2E1 activity. These data suggest that hypothermia is mitigating potential tissue damage in organs involved in drug metabolism in the sub-acute phase after injury. Future studies will provide greater insight into the time-course of these alterations as well as other mechanisms behind the changes observed in this study. Although controlled clinical trials need to be employed in order to fully understand the effects of hypothermic therapy on the disposition of medications in the CA patient population, these data imply that clinically significant hypothermia-drug interactions are a likely factor to consider in the therapeutic course of these patients.

4.0 CHAPTER 4

Mild and moderate hypothermia alters CYP2E1 and CYP3A4 enzyme kinetics in human hepatic microsomes

INTRODUCTION

In Chapter 2, we showed that hypothermia produces a significant decrease in the Cl_s of the CYP2E1 substrate CZN, in CA rats under hypothermic temperatures vs. CA rats maintained under normothermic temperatures (Tortorici, Kochanek et al. 2006). Furthermore, we demonstrated that the mechanism of this decrease in the Cl_s of CZN was a decrease in the affinity of CYP2E1 for CZN. This work also investigated the application of using *in vitro-in vivo* scaling techniques to accurately predict hypothermia-mediated *in vivo* alterations from an *in vitro* enzyme kinetic analysis using rat liver microsomes. This work showed that we could predict the ~2-fold decrease in the Cl_s of CZN observed in the CA rat from *in vitro* enzyme kinetic analysis. Therefore, we set out to expand upon these results in order to identify the effects of mild and moderate hypothermia on CYP450-mediated metabolism in human liver microsomes using *in vitro* techniques to predict potential changes in patients during hypothermia. The objectives of this study were to evaluate the effects of hypothermia on the *in vitro* enzyme kinetics of CYP2E1 and CYP3A4 using CZN and testosterone (TST) as probe substrates, respectively, in 1) pooled human liver microsomes and 2) individual human liver microsomes.

4.1 METHODS

4.1.1 Materials and Chemicals

Pooled human liver microsomes were purchased from XenoTech (Lenexa, KS) with a sample number of 50 donors per batch. Chlorzoxazone (CZN), 6-hydroxychlorzoxazone (6-OH CZN), umbelliferone, testosterone (TST) were purchased from Sigma Aldrich (St Louis, MO.) 6 β -hydroxytestosterone (6 β -OH TST) and 11 β -hydroxytestosterone (11 β -OH TST) were purchased from Steraloids (Newport,RI). Organic solvents were purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise specified.

4.1.2 Individual Human Liver Microsomal Preparation

Liver tissue was collected from 4 human liver donors and microsomes were prepared as previously described by Rockich and Blouin (1999) via differential centrifugation in a Beckman L8-70 ultracentrifuge (Beckman Instruments, Fullerton, CA). Livers were obtained from donors who had consented for use of liver tissue for research purposes. IRB approval for use of tissue for *in vitro* studies was also obtained by the University of Pittsburgh IRB. Total protein concentration was determined as described by (Lowry, Rosebrough et al. 1951).

4.1.3 CYP2E1 Kinetic Analysis

Pooled and individual human liver microsomes were used in these experiments and analysis was performed as outlined in Chapter 2 with some modifications. Briefly, microsomal incubations

contained 400 µg microsomal protein and 1 mM NADPH. CZN was added to microsomal samples in increasing concentrations of 50, 75, 100, 200, 300, 400, and 500 µM. These samples (n= 6 for pooled human hepatic microsomes and n=4 for individual human hepatic microsomes) were incubated for 20 minutes at 37°C, 33°C, or 30°C in a circulating water bath. The reaction was stopped by adding 50 µl of 42.5% o-phosphoric acid, followed by the addition of the internal standard, umbelliferone (78µM).

6-OH CZN was measured by a modified HPLC method described by Rockich and Blouin as described in Chapter 2, subsection 2.1.3 (Rockich and Blouin 1999). Briefly, after the incubation and the addition of internal standard, 5 ml of diethyl ether was added to each sample. The organic phase was transferred and evaporated to dryness under a stream of nitrogen. Samples were then reconstituted with 200 µl of mobile phase A, consisting of 78% of a 0.25% acetic acid solution and 22% acetonitrile, and 20 µl was injected onto the HPLC system. Mobile phase B consisted of 95% acetonitrile and 5% of a 0.25% acetic acid solution and a gradient was established. Chlorzoxazone, umbelliferone, and 6-OH CZN were separated using a Nova Pak C-18 reverse phase (5µm, 3.9 X 150mm) column (Waters, Millford, MA). The HPLC system consisted of a Waters 2695 with a 2487 UV detector, set at 287 nm for CZN and 296 nm for 6-OH CZN. The ratio of 6-OH CZN to umbelliferone was used for quantitative analysis. The C.V. was less than 10% for this assay

4.1.4 CYP3A4 Enzyme Kinetic Analysis

Pooled and individual human liver microsomes were used in these experiments. Microsomal incubations contained 400 µg microsomal protein and 1 mM NADPH. Testosterone was added to microsomal samples in increasing concentrations of 6.25, 12.5, 25, 50, 75, 100, 150, 200, and

250 μM . These samples (n= 6 for pooled human hepatic microsomes and n=4 for individual human hepatic microsomes) were incubated for 10 minutes at 37°C, 33°C, or 30°C in a circulating water bath. The reaction was stopped by placing the samples on ice, followed by the addition of the internal standard, 11 β -OH TST (12.5 μM).

6 β -OH TST was measured by a modified HPLC method described by Sonderfan *et al*, as previously mentioned in Chapter 3, subsection 3.1.4 (Sonderfan, Arlotto et al. 1987). Briefly after the incubation and the addition of internal standard, 5 ml of dichloromethane was added to each sample. The organic phase was transferred and evaporated to dryness under a stream of nitrogen. Samples were then reconstituted with 200 μl of 50:50 methanol: ddH₂O. Mobile phase A consisted of filtered ddH₂O and Mobile phase B consisted of filtered methanol at a flow rate of 1.2 ml/min and 20 μl was injected onto the HPLC system. 6 β -OH TST, 11 β -OH TST, and TST were separated using a Nova Pak C-18 reverse phase (5 μm , 3.9 X 150mm) column (Waters, Millford, MA). The HPLC system consisted of a Waters 2695 with a 2487 UV detector, set at 254 nm. The ratio of 6 β -OH TST to 11 β -OH TST was used for quantitative analysis. The C.V. was less than 10% for this assay

4.1.5 CYP2E1 and CYP3A4 Enzyme Kinetic Analysis

A formal Michaelis-Menten kinetic analysis was performed from the formation rates of 6-OH CZN and 6 β -OH TST using WinNonlin version 4.1 (Pharsight Corp, Mountain View, CA). Maximum velocity (V_{max}) and Michaelis-Menten constant (K_m) were calculated via non-linear regression of the raw data. The data was then linearized for visual inspection, via an Eadie-Hofstee plot.

The classic Michaelis-Menten equation was used for CYP2E1 pooled and individual human liver microsomal analysis:

$$v = \frac{V_{\max} \times [S]}{K_m + [S]} \quad (1)$$

where v is the velocity and $[S]$ is the substrate concentration. The *in vitro* Cl_{int} for CYP2E1 was calculated using the equation:

$$Cl_{\text{int}} = \frac{V_{\max}}{K_m} \quad (2)$$

For CYP3A4 pooled microsomal analysis the following equation was used due to the sigmoidal shape of the saturation curves (Houston and Kenworthy 2000):

$$v = \frac{V_{\max} * [S]^n}{(K_m + [S])^n} \quad (3)$$

where n is the Hill coefficient. For sigmoidal models, Cl_{intmax} was calculated using the following equation as described by Houston and Kenworthy (Houston and Kenworthy 2000):

$$Cl_{\text{intmax}} = \frac{V_{\max}}{K_m} \times \frac{(n-1)}{n(n-1)^{1/n}} \quad (4)$$

The analysis of CYP3A4 and CYP2E1 from individual human liver donors utilized a nonlinear mixed effects modeling approach (as implemented in NONMEM®) to obtain the V_{\max} and K_m parameters for each human liver donor. This approach preserved the within and between

individual variance present in this longitudinal measurement data across the assessed temperatures. The nonlinear mixed effects modeling approach utilizes every data point to determine a population “average” and variance for the group and simultaneously determines individual specific estimate of parameters based on these population characteristics. Therefore, balance in the groups is not as critical (weighting across individual contributions to this model is based on the data contributed as well as the informativeness of the data to a particular parameter). In this approach, a population average and variance that in the case of the exponential model comprised a population average V_{max} and K_m and their variance between individuals in this group. Initially, the model did not account for temperature and lumped estimates were determined. Subsequent to this, each temperature was tested as a covariate in the model to examine whether or not that temperature had improved the description of the data significantly. The effect on the statistical fitness of the model was determined by the change in the Objective Function returned by NONMEM (see statistical section below for detail).

The *in vitro* Cl_{int} or $Cl_{int,max}$ was used to estimate the *in vivo* Cl_S in a human for CYP2E1 and CYP3A4 as described by (Obach, Baxter et al. 1997). The following equation explains the predicted *in vivo* parameters from *in vitro* estimates:

$$Cl_{int} \times \frac{\text{mg microsomal protein}}{\text{gram liver}} \times \frac{\text{liver weight (g)}}{\text{body weight (kg)}} \times \frac{\text{incubation volume (ml)}}{\text{mg microsomal protein}} \quad (5)$$

Where Cl_{int}' is the estimated *in vivo* intrinsic clearance. The estimated Cl_S was then calculated by multiplying the Cl_{int}' and the fraction unbound (f_u). All results are given as means \pm S.D.

4.1.6 Statistical Analysis

For analysis of pooled human microsomal data a one-way ANOVA with a Tukey's post hoc test was utilized. For individual human liver microsomal data, the threshold for determining the significance was based on the Objective Function returned by the NONMEM program. This Objective Function approximates -2 times the log likelihood and thus provides a means of statistically comparing the temperature effects across models. The difference in this term between nested models approximates a χ^2 distribution (Edwards 1972). A change in the objective function of at least 3.84 for 1 degree of freedom (i.e., one change in a model versus another for nested models) was used as a threshold for determining statistical significance at the $\alpha=0.05$ level when using the first-order conditional estimation algorithm with interaction.

Data are presented as mean \pm S.D. All non-NONMEM analyses were conducted using the software program Prism (GraphPad Software, San Diego, CA).

4.2 RESULTS

4.2.1 Effect of Temperature on CYP2E1 Enzyme Kinetics in Pooled Human Liver Microsomes

As shown in **Table 7**, there were significant decreases in the V_{\max} of CZN in microsomes incubated at both 33°C (1.37 ± 0.152 nmol/mg/min;***p<0.001), and 30°C (1.86 ± 0.456 nmol/mg/min;*p<0.05) when compared to 37°C (2.41 ± 0.245 nmol/mg/min). There was also a significant decrease in the V_{\max} of CZN between microsomes incubated at 33°C compared to 30°C; ‡p<0.05. **Table 7** also shows that the K_m of CYP2E1 showed no difference in microsomes

incubated at 37°C and 33°, 80.6 ± 23.6 μM and 85.4 ± 18.5 μM, respectively. However, when human liver microsomes were incubated at 30°C, the K_m was significantly increased, 159 ± 48.2 μM; $^{**,\ddagger\ddagger}p<0.01$, when compared to both 37°C and 33°C. These results are more easily visualized in an Eadie-Hofstee plot depicted in **Figure 20, Panel B**. These parameters were used to calculate Cl_{int} using Equation 2 and data demonstrated a decrease in the Cl_{int} of CYP2E1 at 33°C (0.0164 ± 0.00206 ml/min/mg protein; $^{***}p<0.001$) and 30°C (0.0128 ± 0.00534 ml/min/mg protein; $^{***}p<0.001$), when compared to 37°C (0.0314 ± 0.00715 ml/min/mg protein). After performing an *in vitro-in vivo* correlation, using Equation 5, the estimated Cl_s of CZN was 531.07 ± 120 ml/min at 37°C and significantly decreased at 33°C (276.74 ± 34 ml/min; $^{***}p<0.001$) and 30°C (215.65 ± 90; $^{***}p<0.001$).

4.2.2 Effect of Temperature on CYP2E1 Enzyme Kinetics in Human Liver Microsomes from Individual Liver Donors

Each individual human liver microsome incubated at the 3 different temperatures showed a rank order decrease from 37°C to 33°C to 30°C in the Cl_{int} of CYP2E1 as depicted in **Figure 21**. Each individual demonstrated a decrease in Cl_{int} of CZN at 33°C (22.95 ± 0.001% of 37°C) and

Table 7: CYP2E1 enzyme kinetic parameter estimates in pooled human liver microsomes incubated at 37°C, 33°C, or 30°C

	37 Degrees	33 Degrees	30 Degrees
Vmax (nmol/mg/min)	2.41 ± 0.25	1.37 ± 0.15 ***,‡	1.86 ± 0.46 *
Km (uM)	80.59 ± 23	85.36 ± 18	159.35 ± 48 **,‡‡
Clint (ml/min/mg protein)	0.031 ± 0.0072	0.016 ± 0.0021 ***	0.013 ± 0.0053 ***
Cl_s estimated (ml/min)	531.07 ± 120	276.74 ± 34 ***	215.65 ± 90 ***

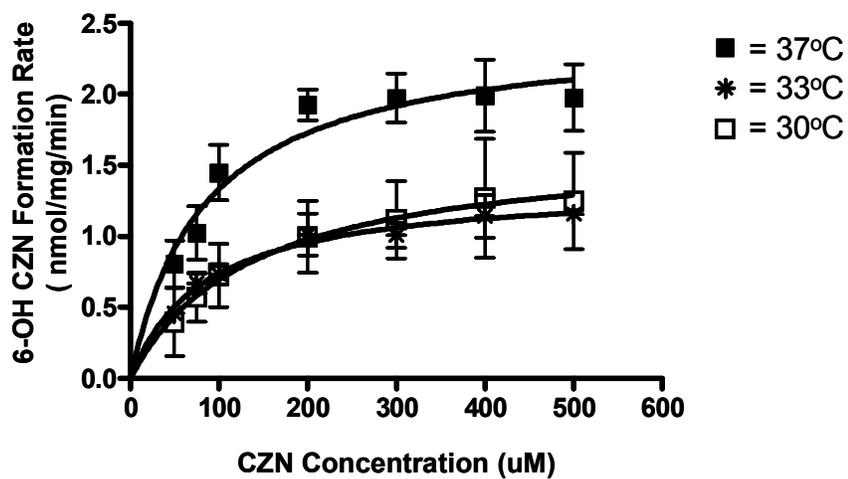
$p<0.05$ * , \ddagger $p<0.01$ ** , $\ddagger\ddagger$, $p<0.001$ ***

* = statistically significant compared to 37°C

\ddagger = statistically significant differences between 33°C and 30°C

Abbreviations: Vmax, maximal velocity; Km, Michaelis-Menten constant; Clint, intrinsic clearance

A.



B.

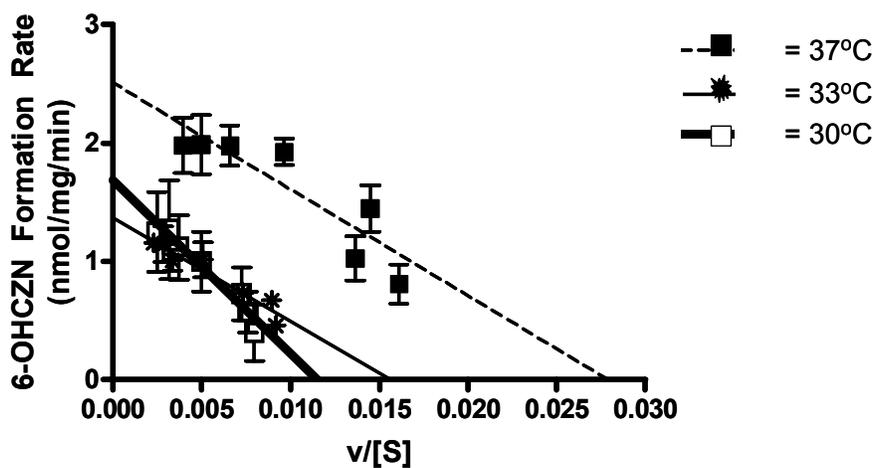


Figure 20: Michaelis-Menten saturation curves in human liver microsomes for CYP2E1

Panel A depicts saturation curves in human liver microsomes for CYP2E1 incubated at 37°C, 33°C, and 30°C, n=6. Panel B depicts an Eadie-Hofstee plot constructed from the non-linear regression analysis of the raw data reported in Table 7. Decreases in V_{max} can be observed upon visual inspection of the Eadie-Hofstee plot, as all three lines have different y-intercepts. The change in K_m at 30°C can be observed as the slope of the line compared to 33°C and 37°C.

30°C ($55.67 \pm 0.001\%$ of 37°C). The individual estimates here were taken from the Bayesian estimates returned from the NONMEM model using the FOCE option. The population estimates for V_{max} demonstrate a significant decrease at 30°C, (0.697 nmol/mg/min, 36.3% standard error (se); $p < 0.0001$, chi-square test, $df=2$) compared to 33°C (0.901 nmol/mg/min, 34.2% se) and 37°C (0.929 nmol/mg/min, 42.1% se). There was a trend towards a decrease in the V_{max} at 33°C compared to 37°C; however, it did not demonstrate significance. The population estimates for K_m demonstrated a significant rank order increase as temperature decreases ($p < 0.0001$, chi-square test, $df=2$). These estimates were 70.6 μM (6.9% se) at 37°C, 89.6 μM (9.4% se) at 33°C, and 120 μM (12.5% se) at 30°C. Inspection of the individual sample parameters demonstrated similar results in the V_{max} and K_m as temperature decreases, as shown in **Table 8**. Inter-individual variability was estimable for V_{max} (~80%) but not for the K_m . Individual 1 demonstrated a decrease in the estimated Cl_S at 33°C and 30°C, 338 ml/min and 194 ml/min, respectively, when compared to 37°C, 438 ml/min. Individual 2 demonstrated a decrease in the estimated Cl_S at 33°C and 30°C, 263 ml/min and 151 ml/min, respectively, when compared to 37°C, 341 ml/min. Individual 3 demonstrated a decrease in the estimated Cl_S at 33°C and 30°C, 188 ml/min and 108 ml/min, respectively, when compared to 37°C, 244 ml/min. Individual 4 demonstrated a decrease in the estimated Cl_S at 33°C and 30°C, 51.3 ml/min and 29.5 ml/min, respectively, when compared to 37°C, 66.6 ml/min.

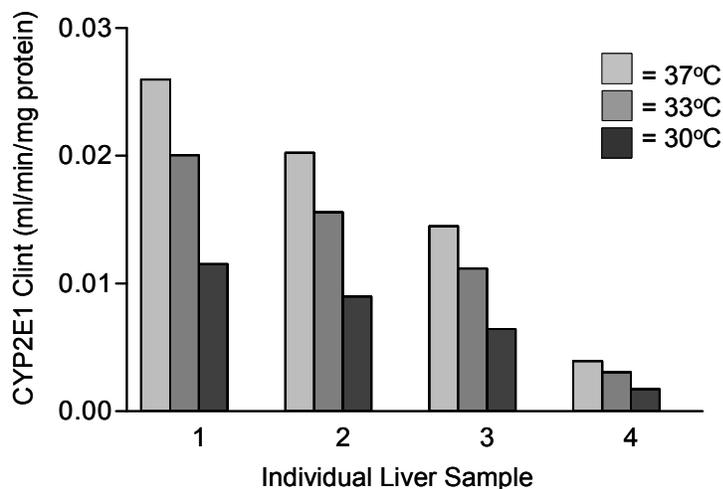


Figure 21: Intrinsic clearance of CYP2E1-mediated metabolism in individual human liver microsomes

Using the *in vitro* parameter estimates (V_{max} and K_m) obtained from the NONMEM analysis, Cl_{int} was calculated by the equation V_{max}/K_m . This data depicts a rank order decrease in the Cl_{int} of CYP2E1 incubated at 33°C or 30°C, compared to 37°C.

Table 8: CYP2E1 enzyme kinetic parameters estimates in individual human liver microsomes incubated at 37°C, 33°C, or 30°C

Sample (temp)	V_{max} (nmol/mg/min)	K_m (μM)	Cl_{int}' (ml/min/kg)	Cl_s' (ml/min)
1 (37 degrees)	1.83	70.6	58.4	438
1 (33 degrees)	1.79	89.6	45.0	338
1 (30 degrees)	1.38	120	25.9	194
2 (37 degrees)	1.43	70.6	45.5	341
2 (33 degrees)	1.40	89.6	35.1	263
2 (30 degrees)	1.07	120	20.2	151
3 (37 degrees)	1.02	70.6	32.6	244
3 (33 degrees)	1.00	89.6	25.1	188
3 (30 degrees)	0.767	120	14.4	108
4 (37 degrees)	0.278	70.6	8.87	66.6
4 (33 degrees)	0.272	89.6	6.84	51.3
4 (30 degrees)	0.209	120	3.93	29.5

Abbreviations: V_{max} , maximal velocity; K_m , Michaelis-menten constant; Cl_{int}' , estimated intrinsic clearance; Cl_s' , estimated systemic clearance

4.2.3 Effect of Temperature on CYP3A4 Enzyme Kinetics in Pooled Human Liver Microsomes

As depicted in **Table 9**, there were significant decreases in the V_{max} of CYP3A4 in microsomes incubated at both 33°C (3.56 ± 0.707 nmol/mg/min), and 30°C (3.87 ± 0.307 nmol/mg/min;*** $p < 0.001$) when compared to 37°C (9.28 ± 1.2 nmol/mg/min). **Table 9** also demonstrates that the K_m of CYP3A4 shows no significant difference in microsomes incubated at 37°C, 33°C, and 30°C, 46.9 ± 17.6 μ M, 35.7 ± 17.3 μ M, and 22.4 ± 5.35 μ M, respectively. **Figure 22, Panel B** demonstrates a hooked Eadie-Hofstee plot for all three incubation temperatures. The Hill coefficient demonstrated no differences between temperatures. These changes in V_{max} , K_m and the Hill coefficient resulted in no changes in the $Cl_{int,max}$ of CYP3A4 at all three temperatures. After performing an *in vitro-in vivo* correlation, the estimated Cl_s of testosterone did not differ based on temperature.

Table 9: CYP3A4 enzyme kinetic parameter estimated in pooled human liver microsomes incubated at 37°C, 33°C, or 30°C

	37°C	33°C	30°C
Vmax (nmol/mg/min)	9.28 ± 1.20	3.56 ± 0.707 ***	3.87 ± 0.307 ***
Km (uM)	46.9 ± 17.6	35.7 ± 17.3	22.4 ± 5.35
Hill coefficient	1.56 ± 0.25	1.58 ± 0.18	1.75 ± 0.43
Clint max(ml/min/mg protein)	0.0306 ± 0.0136	0.0172 ± 0.00832	0.0284 ± 0.0110
Cl_s estimated (ml/min)	713 ± 149	398 ± 186	605 ± 115

$p < 0.001$ ***

Abbreviations: V_{max} , maximal velocity; K_m , Michaelis-menten constant; Cl_{int} , intrinsic clearance; Cl_s , estimated systemic clearance.

4.2.4 Effect of Temperature on CYP3A4 Enzyme Kinetic in Human Liver Microsomes from Individual Donors

Each individual human liver microsome incubated at 3 different temperatures showed a rank order decrease from 37°C to 33°C to 30°C in the Cl_{int} of CYP3A4 as depicted in **Figure 23**. Each individual demonstrated a decrease in the Cl_{int} of CYP3A4 at 33°C ($13.65 \pm 0.001\%$ of 37°C) and 30°C ($27.09 \pm 0.001\%$ of 37°C). The population estimates for V_{max} demonstrated a significant decrease as temperature decreases ($p < 0.0001$, chi-square, $df=2$). These estimates were at 5.93 nmol/min/mg (48.5% se) at 37°C, 5.12 nmol/mg/min at 33°C (47.9% se) and 4.32 nmol/mg/min (46.5% se) at 30°C. The population estimates for K_m were not distinguishable between temperatures. Inspection of the individual sample parameters demonstrated similar results in the V_{max} and K_m as temperature decreases as depicted in **Table 10**. Inter-individual variability was estimable for V_{max} (~98%) but not for the K_m . Individual 1 demonstrated a decrease in the estimated Cl_S at 33°C and 30°C, 1223 ml/min and 1032 ml/min, respectively, when compared to 37°C, 1416 ml/min. Individual 2 demonstrated a decrease in the estimated Cl_S at 33°C and 30°C, 558.8 ml/min and 471.8 ml/min, respectively, when compared to 37°C, 647.1 ml/min. Individual 3 demonstrated a decrease in the estimated Cl_S at 33°C and 30°C, 65.99 ml/min and 55.72 ml/min, respectively, when compared to 37°C, 76.43 ml/min. Individual 4 demonstrated a decrease in the estimated Cl_S at 33°C and 30°C, 9.484 ml/min and 8.008 ml/min, respectively, when compared to 37°C, 10.98 ml/min.

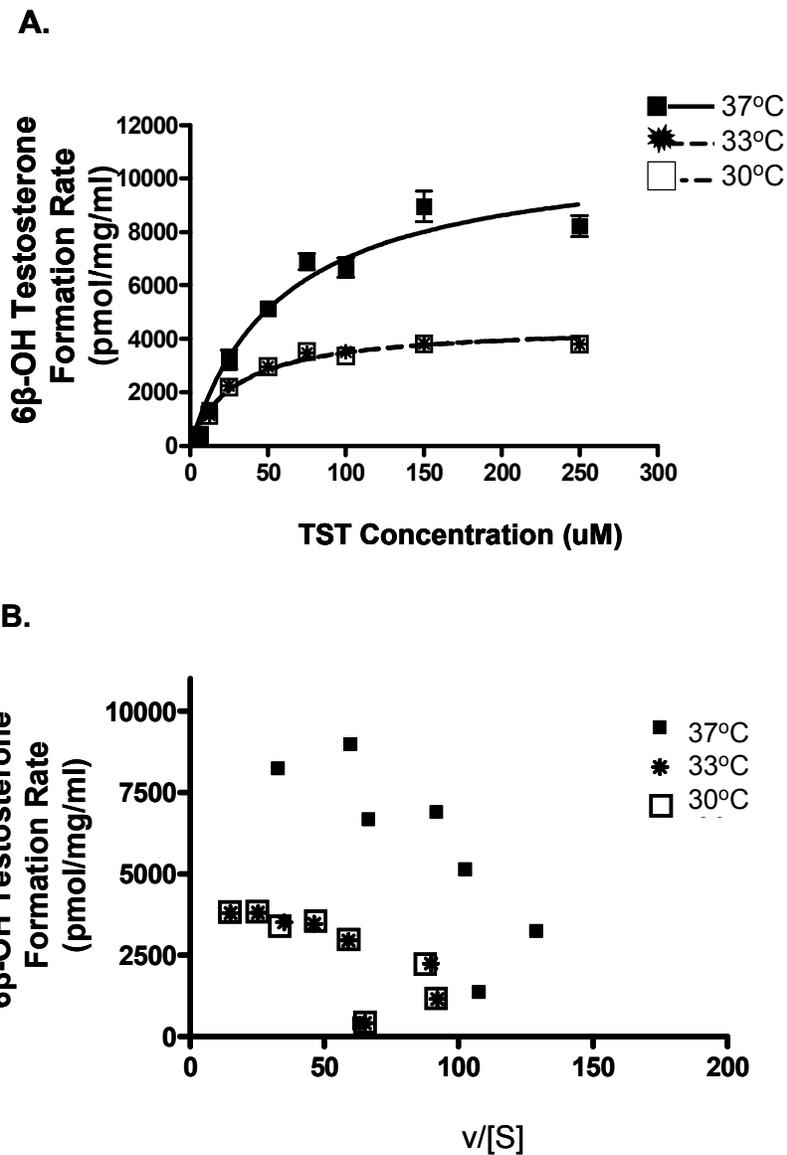


Figure 22: Michaelis-Menten saturation curves in human liver microsomes for CYP3A4

Panel A depicts the Michaelis-Menten saturation curves in human liver microsomes for CYP3A4 incubated at 37°C, 33°C, or 30°C, n=6. Panel B depicts an Eadie-Hofstee plot constructed from the non-linear regression analysis of the raw data reported in Table 9. Decreases in V_{max} can be observed upon visual inspection of the Eadie-Hofstee plot, as all three lines have different y-intercepts.

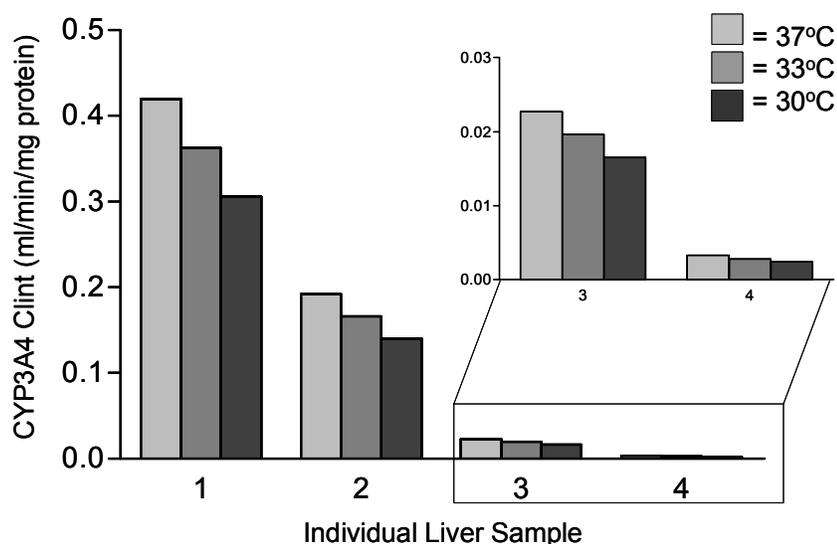


Figure 23: Intrinsic clearance of CYP3A4-mediated metabolism in individual human liver microsomes

Using the *in vitro* parameter estimates (V_{max} and K_m) obtained from NONMEM analysis, Cl_{int} was calculated by the equation V_{max}/K_m . This data depicts a rank order decrease in the Cl_{int} of CYP3A4 incubated at 33°C or 30°C, compared to 37°C. The inset allows for inspection of liver samples 3 and 4 on a smaller scale.

Table 10: CYP3A4 enzyme kinetic parameters estimates in individual human liver microsomes incubated at 37°C, 33°C, or 30°C

Sample (temp)	Vmax (nmol/mg/min)	Km (μ M)	Clint' (ml/min/kg)	Cl _s ' (ml/min)
1 (37 degrees)	15.2	36.2	944	1416
1 (33 degrees)	13.1	36.2	815	1223
1 (30 degrees)	11.1	36.2	688	1032
2 (37 degrees)	11.7	61.1	431	647.1
2 (33 degrees)	10.1	61.1	373	558.8
2 (30 degrees)	8.54	61.1	315	471.8
3 (37 degrees)	1.12	49.6	51.0	76.43
3 (33 degrees)	0.969	49.6	44.0	65.99
3 (30 degrees)	0.818	49.6	37.1	55.72
4 (37 degrees)	0.204	62.8	7.32	10.98
4 (33 degrees)	0.177	62.8	6.32	9.484
4 (30 degrees)	0.149	62.8	5.34	8.008

Abbreviations: Vmax, maximal velocity; Km, Michaelis-menten constant; Clint', estimated intrinsic clearance; Cl_s, estimated systemic clearance

4.3 DISCUSSION

Our results demonstrate three novel findings. First, mild and moderate hypothermia decreased the Cl_{int} of CYP2E1-mediated metabolism of CZN however, did not affect Cl_{intmax} of CYP3A4-mediated metabolism of TST in pooled human hepatic microsomes. When an *in vivo-in vitro* correlation analysis was performed, the estimated Cl_s for CZN demonstrated a decrease at both mild and moderate hypothermia and no difference in the estimated Cl_s of TST was observed between temperatures. Second, temperature-mediated alterations were due to decreases in the V_{max} of both CYP3A4 and CYP2E1 and an increase in the K_m of the CYP2E1 enzyme. Third, individual human liver microsomes incubated at both mild and moderate hypothermic temperatures demonstrated a rank order decrease in the Cl_{int} of both CYP2E1 and CYP3A4.

We demonstrated that mild (33°C) and moderate hypothermia (30°C) decreased the Cl_{int} of CYP2E1, ~1.9-fold and ~2.5-fold, respectively, compared to 37°C in pooled human liver microsomes. This further translated into a decrease at both mild and moderate hypothermic temperatures in the estimated Cl_s of CZN. This is consistent with our previous finding demonstrating a ~1.7-fold decrease at 30°C compared to incubation at 37°C in the Cl_{int} of CYP2E1 in rat liver microsomes (Tortorici, Kochanek et al. 2006). Together, these results suggest that both mild and moderate hypothermia decreases the intrinsic ability of CYP2E1 to metabolize the probe substrate CZN.

This study also showed that when pooled human liver microsomes were incubated at 33°C and 30°C, the V_{max} of CYP2E1 was decreased ~1.7 and ~1.3-fold, respectively, compared to 37°C. This is in contrast to our previous finding in which the V_{max} of CYP2E1 in rat liver microsomes did not differ when incubated at 30°C versus 37°C (Tortorici, Kochanek et al. 2006).

Changes in V_{\max} represent a change in the capacity of the enzyme, more specifically alterations in the functionality of the heme group. It has been postulated that cooling decreases enzymatic processes by a factor of approximately 1.5 to 3 per 10°C temperature decreases (Zachariassen 1991). The rate of the redox reaction of the iron atom present in the heme prosthetic group may be affected by mild and moderate reductions in temperature. Furthermore, these data provide evidence that temperature may differentially affect human and rat liver CYP450 metabolism.

We observed that the K_m of CYP2E1 in pooled microsomes incubated at 30°C significantly increased compared to incubations conducted at 37°C and 33°C. The observed increase in K_m due to hypothermic conditions is consistent with a study by McAllister *et al.*, who demonstrated that when propranolol and verapamil were incubated at 26°C versus 37°C in rat liver microsomes, the K_m was significantly increased at the lower temperature (McAllister and Tan 1980). As outlined in Chapter 2, when CZN was incubated at 30°C in rat liver microsomes, a ~2.1-fold increase in the K_m was observed compared to microsomes incubated at 37°C (Tortorici, Kochanek et al. 2006). The ability of an enzyme to bind a substrate depends on a specific geometry which allows the interaction of the active site and substrate (Fields 2001). Variation in the flexibility of the active site of the enzyme due to moderately hypothermic temperatures could be a potential cause of the difference we observed in our K_m values of CYP2E1. Collectively, these results demonstrate that mild and moderate hypothermic temperatures reduce the capacity of CYP2E1 in human liver microsomes. Furthermore, at moderate hypothermia, the affinity of CYP2E1 for CZN is also decreased.

Testosterone is metabolized by CYP3A4 and has demonstrated atypical enzyme kinetics in previous reports (Korzekwa, Krishnamachary et al. 1998; Shou, Mei et al. 1999; Houston and Kenworthy 2000). This observation is evident upon visual inspection of the Eadie-Hofstee plot,

Figure 20, Panel B, which shows a hooked shape. Testosterone has been shown to exhibit autoactivation, which is defined as an increase in the reaction velocity as more molecules of TST bind to the active site (Korzekwa, Krishnamachary et al. 1998; Houston and Kenworthy 2000). Our data demonstrated that mild and moderate hypothermia did not affect the Cl_{intmax} of TST. We showed that under both mild and moderate hypothermic conditions, the V_{max} was decreased ~2.6 and ~2.3 fold respectively. As mentioned above, this could be caused by hypothermic temperatures decreasing the rate of redox reaction of the iron atom of the heme prosthetic group. We did not observe any differences in the K_m or Hill coefficient at 33°C or 30°C, which is in contrast to CYP2E1. This discrepancy between CYP3A4 and CYP2E1 could be related to the difference in the size of the active site. It is known that CYP2E1 has a small binding pocket, with one binding site for CZN (Tan, White et al. 1997). In contrast, CYP3A4 has a large binding pocket, with multiple binding sites for TST (Scott and Halpert 2005). For CYP3A4, even though the flexibility of the binding pocket might be altered by hypothermic conditions (Fields 2001), the fact that TST has multiple binding sites which demonstrate positive co-operativity, could be an explanation of why a decrease in temperature does not change the affinity of CYP3A4 for TST (Houston and Kenworthy 2000). Together, these results show that hypothermia produces a consistent decrease in the capacity of multiple isoforms of CYP450; however our findings allude to the fact that hypothermia-mediated alterations may be isoform specific, depending on the structure of the substrate binding pocket.

Lastly, we performed these analyses in individual human liver donor samples to confirm our findings observed in pooled microsomes. For CYP2E1, we demonstrated a significant rank order increase in K_m and decrease in V_{max} from 37°C to 33°C to 30°C. These differences resulted in significant decreases in Cl_{int} at both 33°C and 30°C compared to 37°C, which is consistent

with data generated from the pooled liver microsomes. For CYP3A4 in individual human liver donor microsomes, we demonstrated a significant rank order decrease in the V_{\max} from 37°C to 33°C to 30°C. However, the K_m was not significantly different between temperatures. Due to the dramatic decreases in V_{\max} , these differences resulted in significant decreases in the Cl_{int} at both 33°C and 30°C compared to 37°C, which is in contrast to the data generated from pooled liver microsomes. These differences between CYP isoforms (CYP2E1 and CYP3A4) are possibly related to the differences in the size of the active site as described above (Houston and Kenworthy 2000; Scott and Halpert 2005).

There are several possible limitations of our study. First, the results from the CYP3A4 pooled microsomal analysis revealed an unexpected variability in the K_m values, which could be due to the limited number of concentrations analyzed around the K_m . This could limit some of the conclusions; however, when the individual human liver microsomes were analyzed using NONMEM, the K_m across temperatures could not be differentiated, lending support to the results from the pooled microsomal analysis. Second, our results for Cl_{int} of CYP3A4 are inconsistent between pooled and individual human liver microsomes. This could be due to the small sample size in the individual human liver microsome (n=4) analysis compared to pooled human liver microsomes (n=50), demonstrating greater decreases in V_{\max} compared to pooled microsomes. It is also noteworthy to mention that the data from the individual liver microsomes fit to hyperbolic kinetic model and the data from the pooled liver microsomes fit to sigmoidal kinetic model, which could account for the differences observed. Lastly, we only investigated two CYP isoforms, CYP2E1 and CYP3A4. It is possible that other CYP isoforms behave differently than the two isoforms investigated and future studies are in place to evaluate these effects.

4.4 CONCLUSION

This study reports that incubating pooled and individual human liver microsomes at mild (33°C) and moderate (30°C) hypothermic temperatures alters the enzyme kinetic profile of CYP2E1, specifically decreases in the Cl_{int} . In pooled microsomes, we demonstrated alterations in enzyme kinetics of CYP3A4, specifically decreases in the V_{max} . Individual human liver microsomal analysis for both CYP2E1 and CYP3A4 demonstrated significant decreases in Cl_{int} at mild and moderate hypothermic temperatures. The magnitude of these changes in Cl_{int} parallel previously published clinical data. Fukuoka *et al* reported ~5-fold increase in plasma concentrations of midazolam (CYP3A4 substrate) in traumatic brain injured patients treated with mild hypothermia (33°C) compared to normothermia (Fukuoka, Aibiki et al. 2004). Iida *et al* observed a ~67% decrease in the Cl_S of phenytoin (CYP2C9) in traumatic brain injured patients treated with mild hypothermia (~34°C) compared to normothermia (Iida, Nishi et al. 2001). Similarly, Caldwell *et al.* demonstrated a ~11% decrease per degree Celsius in the Cl_S of vecuronium in patients (Caldwell, Heier et al. 2000). Future studies involving additional CYP450 isoforms and other pathways of metabolism are ongoing to fully elucidate the effects of hypothermic therapy on drug disposition and metabolism.

5.0 CHAPTER 5

The Effects of Mild Hypothermia on Midazolam Pharmacokinetics in Normal Healthy
Subjects: A Pilot Study

INTRODUCTION

It has been well documented that therapeutic hypothermia (32°C-35°C) decreases neurological damage in patients experiencing out-of-hospital CA (Bernard, Gray et al. 2002; Group 2002). Due to the positive results from these two landmark clinical trials, interest in therapeutic hypothermia has expanded its use to other ischemic insults, such as in stroke and TBI, with the goal of reducing neurological damage. Animal models have been used to refine the use of hypothermia (ie. optimal depth, duration, and method of cooling) in these ischemic insults. Multiple methods of cooling a patient have been developed in order to achieve goal core temperature quickly. One method is surface cooling, which is non-invasive and potentially the safest method for inducing mild hypothermia. However, this technique has a limited ability to quickly bring the patient to target temperature, which has been shown to be critical in the benefit of hypothermia therapy (Nozari, Safar et al. 2006). Additionally, surface cooling requires substantial resources such as large quantities of ice, chemical icepacks, or powered cooling blankets. Another method is endovascular cooling, which delivers chilled saline directly to the body (Al-Senani, Graffagnino et al. 2004). This is a less costly and resource intensive alternative, this method of cooling could be initiated quickly after injury, resulting in more favorable outcomes.

As mentioned above, mild hypothermia is currently under investigation as a potential therapy after stroke. As with CA, the goal core temperature range is 32°C-35°C; however, unlike CA, patient's experiencing a stroke will defend their temperature via the shivering response. The reason for this is that stroke patients' are typically not administered neuromuscular blockers

under general anesthesia. One method of cooling that has been developed to reach goal temperature in stroke patients is to administer benzodiazepines at the same time the infusion of ice cold saline is started, to prevent the shivering response.

Another critical component in the development of a method to cool patients via ice-cold saline infusion is the effect of mild hypothermia on the pharmacokinetics of the drugs which are commonly employed in these patients. The majority of these medications are hepatically eliminated by the CYP450 enzyme system, including sedatives and anesthetics such as midazolam and fentanyl, both metabolized by CYP3A4. The work presented throughout this dissertation has demonstrated that moderate to mild hypothermic temperatures alter CYP450-mediated metabolism. In Chapter 2, we demonstrated a ~2-fold decrease in the Cl_s of CYP2E1 for CZN, in a CA rat model and the mechanism behind this alteration is due to hypothermia-mediated decrease in the affinity of CYP2E1 for CZN. In Chapter 4, we further demonstrated in human liver microsomes that both mild and moderate hypothermia decreases the capacity of CYP2E1 and CYP3A4 ability to metabolize probe substrates, CZN and TST, respectively. In the development of an endovascular cooling protocol it is also important to optimize the drug dosing regimen. The drug regimen must be comfortable for the patient and suppress or diminish the shivering response without jeopardizing patient safety. Various drugs are available that may suppress the shivering response and yet still be safe to administer to patients who are awake during acute illness. Options include drugs such as meperidine, narcotics, benzodiazepines, and buspirone. In order to optimize this protocol and provide maximum comfort to the patient, it is crucial to understand the effect of cooling on the disposition and metabolism of drugs commonly utilized in these patients. Therefore, the objectives of this pilot analysis were to investigate the

effects of mild hypothermia on the pharmacokinetics of midazolam in normal healthy subjects after ice-cold saline administration.

5.1 METHODS

5.1.1 Subjects

The studies were approved by the biomedical institutional review board of the University of Pittsburgh. Subjects participated in these studies after providing written informed consent. All subjects underwent a screening evaluation including a complete medical history, physical examination, and biochemical analysis. Eligibility criteria include male subjects' age 18-39 years old, without known medical problems. Exclusion criteria included females, any individual with a history of known medical problems including but not limited to; cardiac disease, cardiac arrhythmia or heart murmur, hypotension or hypertension, pulmonary diseases including asthma, diabetes mellitus, thyroid disease, renal disease, liver disease, neurologic disease or seizures, connective tissue disorder or Raynaud's disease, sickle cell disease, anemia, or hematologic disorders, subjects who are HIV positive, subjects taking any medications (including St Johns Wort, MAO inhibitors, antidepressants, cimetidine) with the exception of ibuprofen, or acetaminophen, subjects with history of cardiac disease in family members below the age of forty, allergy to benzodiazepines, subjects currently ill with viral or other infection, subjects who currently smoke, subjects who are morbidly obese ($BMI \geq 36$). All participants were instructed to abstain from alcohol, caffeine-containing products, and grapefruit products for at least 48 hours prior to and during the study period. Three normal healthy male subjects were recruited for study participation.

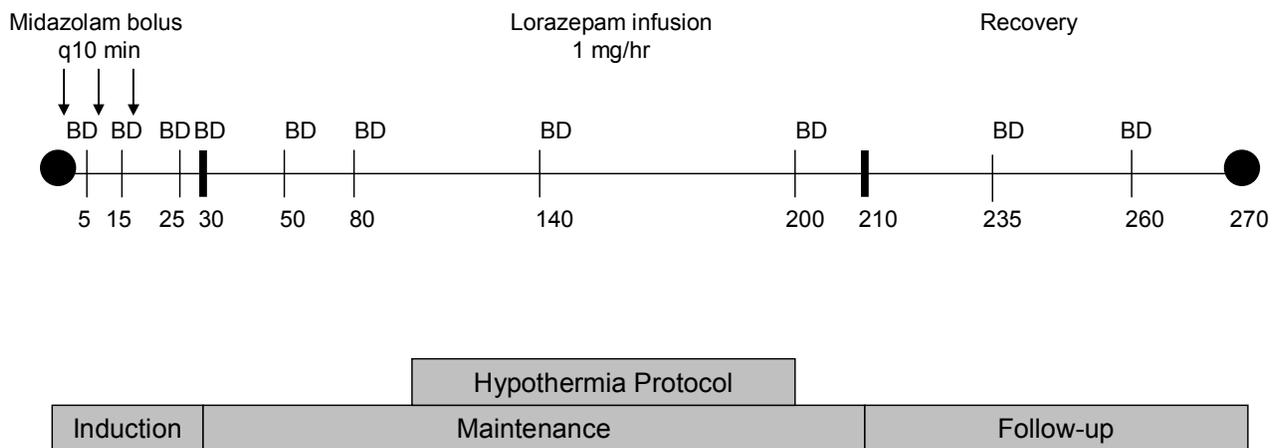
5.1.2 Study Design

This study utilized a prospective, randomized, cross-over study design to determine if therapeutic hypothermia affected the pharmacokinetics of midazolam. Normal healthy male subjects were admitted to the Department of Emergency Medicine the morning after an overnight fast. The subjects were randomized on each study day to receive one of 2 treatment arms as outlined in **Figure 24**. Subjects were to receive, on each consecutive visit, either ice-cold saline (4°C) with sedation or warm saline (37°C) with sedation. Protocols were completed in a temperature and pressure controlled room with an ambient temperature of ~ 23.5°C. Subjects voided and were weighed. Two 18-gauge catheters were placed in superficial vein near the anterior aspect of the elbow for drug administration and blood draws. Each subject ingested a single-use CorTemp (HQ Inc, FL) capsule. A minimum of 30 minutes was provided for the capsule to enter the GI tract. Three consecutive readings within 0.1°C were obtained prior to initiating the protocol. Thermisters were placed on the skin on the opposite side of the infusion arm over the sternal head of the pectoralis major, the supraspinatus, the midpoint of the triceps brachii, and the midpoint of the vastus intermedius muscles to measure skin temperature.



Figure 24: Randomization scheme of sedation and fluid temperature

Each subject was administered 30ml/kg of either warm (37°C) or cold (4°C) saline based on group assignment. Midazolam (MDZ) at a dose of 2 mg IV was administered at the onset of the infusion. Midazolam is a well-established *in vivo* CYP3A4 probe substrate because it is exclusively metabolized by CYP3A4 and has a short half-life. Additional 2 mg doses were administered at 10, and 20 minutes following the first dose. Blood samples (5ml) were collected for analysis of midazolam concentrations as depicted in **Figure 25**. Blood samples were collected at 5, 15, 25, 30, 50, 80, 140, 200, and 260 minutes after the first dose. Blood was separated using a microcentrifuge and plasma was stored at -80°C. Following the saline infusion, a lorazepam infusion of 1mg/hr was initiated to maintain sedation for 3 hours. The dose was titrated to maintain a Ramsey sedation scale rating of 2, which indicates the subject responded to verbal stimuli. At the end of the experimental period, subjects were actively warmed with blankets and heating pads for at least one hour once the maintenance infusion of lorazepam ended. The subjects were asked to void and were weighed. The study physician assessed each subject to determine whether or not the subject could leave the Department of Emergency Medicine.



BD= Blood Draw

Figure 25: Timeline of blood sampling, induction and maintenance of hypothermia.

5.1.3 Sample Analysis

Plasma concentrations of midazolam were determined using a modified and validated HPLC technique previously described by Quintela et al (Quintela, Cruz et al. 2004). Briefly a 0.25-ml aliquot of plasma was combined with 2N sodium hydroxide (0.25 ml), buffer (750 ml) and internal standard (50 ng of [²H₄] -midazolam). Methy-*terbutyl*-ether (5 mL) was added and samples were centrifuged at 3000g for 10 minutes. Samples were evaporated to dryness under a gentle stream of nitrogen at 40°C, and reconstituted in 200 µL of 80:20 0.1% formic acid in ddH₂O:acetonitrile and 20 µL was injected onto the HPLC system. Separation of analytes was achieved on a ThermoFinnigan BetaBasic C₁₈ column and detected using a MSQ single quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA). The mass spectrometer was operated under positive electrospray ionization mode with a probe voltage of 3.0 kV, cone voltage of 80 kV and cone temperature of 350°C for MDZ. Analysis was carried out using selected ion monitoring (SIM) for specific *m/z* 325.9 (midazolam) and 329.9 ([²H₄ -midazolam). The limit of quantification for MDZ in plasma was 2 ng/ml. The within- and between coefficients of variance were less than 15%.

Urine concentrations for validation of 1-hydroxymidazolam (1-OH MDZ) were determined using a modified technique as mentioned above for midazolam. Briefly, a 1 mL aliquot of urine was used and internal standard (50 ng of [²H₄] -midazolam) and methy-*terbutyl*-ether (5 mL) was added and samples were centrifuged at 3000g for 10 minutes. Samples were evaporated to dryness under a gentle stream of nitrogen at 40°C, and reconstituted in 200 µL of 80:20 0.1% formic acid in ddH₂O:acetonitrile and 20 µL was injected onto the HPLC system. Separation of analytes was achieved on a ThermoFinnigan BetaBasic C₁₈ column and detected using a MSQ single quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA). The mass

spectrometer was operated under positive electrospray ionization mode with a probe voltage of 3.0 kV, cone voltage of 65 kV and temperature of 350°C. Analysis was carried out using selected ion monitoring (SIM) for specific m/z 341.9 (1-OH midazolam) and 329.9 ($[^2\text{H}_4]$ - midazolam). The limit of quantification for 1-OH MDZ in urine was 125 ng/ml. The within- and between coefficients of variance were less than 15%.

5.1.4 Pharmacokinetic Analysis

The plasma concentrations of midazolam were fit to a non-compartmental model using the non-linear regression program WinNonlin Professional (version 4.1, PharSight, Mountain View, CA). The area under the plasma concentration-time curve (AUC) was calculated with the use of log-linear trapezoidal methods up to infinity. Systemic MDZ clearance (CL_S) was calculated as the intravenous dose (DOSE_{iv}) divided by the AUC. The amount of 1-OH MDZ in the urine was determined by multiplying the urinary concentration by the volume voided. Other parameters calculated were the terminal elimination rate constant (λ_z), estimated by the linear regression of the terminal phase of the logarithmic plasma-concentration time curve, and the $t_{1/2}$, calculated as $0.693/\lambda_z$.

Data are presented as mean \pm standard deviation (SD). Demographic information and PK parameters between individual subjects when normothermic core temperatures and hypothermic core temperatures using a paired Student's t -test. All statistical calculations were performed with GraphPad Prism v.3.03 (GraphPad Software, Inc., San Diego, CA). A p value < 0.05 was considered statistically significant.

5.2 RESULTS

5.2.1 Validation of a HPLC-MS Assay for Detection of Midazolam in Plasma

5.2.1.1 Chromatographic Separation for Plasma Analysis of Midazolam

Representative chromatograms of plasma samples by SIM mass spectrometric detection are shown in **Figure 26**. Retention times for [$^2\text{H}_4$] –midazolam (internal standard, IS) and MDZ were 10.01 and 10.03 minutes, respectively. The peaks of the compounds of interest were well separated and there was no interference from endogenous compounds during the elution window for the analytes and IS.

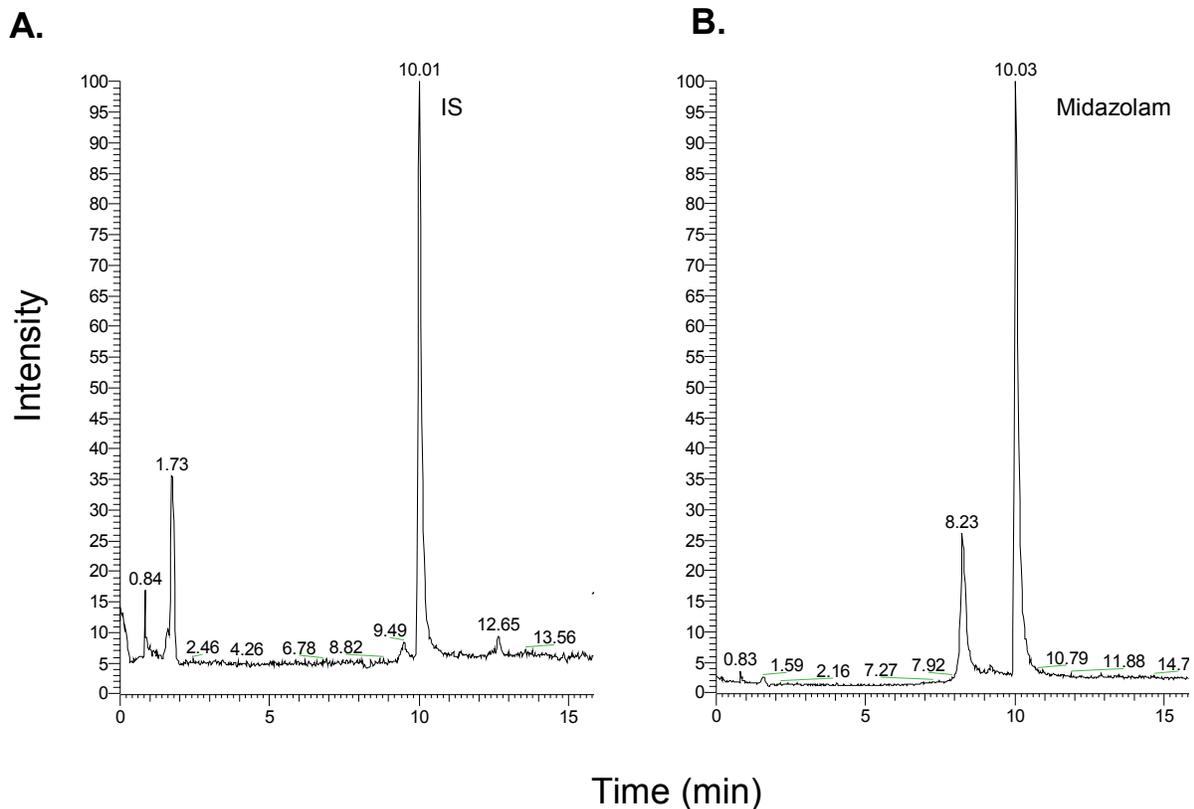


Figure 26: Representative SIM chromatograms of internal standard and midazolam

Internal standard (midazolam D4) in Panel A, and midazolam in Panel B. The LOQ for midazolam is 2 ng/ml

5.2.1.2 Linearity, Precision, and Calibration for Midazolam

Linear calibration curves were obtained for MDZ over the concentration range of 2 to 125 ng/mL. The mean regression equations (\pm SD) were as follows:

$$y = [0.0396 (\pm 0.0007)]x - 0.0091 (\pm 0.018)$$

Correlation coefficients (r) were ≥ 0.989 for standard curves. The lower limit of quantification (LOQ) for MDZ (2 ng/mL or 40 pg on column) demonstrated acceptable reproducibility (%RSD < 5) and a signal-to-noise ratio of 35:1. The intra- and inter-day precision and accuracy were less than $\pm 10\%$ for midazolam (**Table 11**).

Table 11: Intra- and inter-day precision and accuracy for midazolam

	Amount (ng/mL)		% R.S.D.	% Bias
	Added	Observed (mean \pm S.D.)		
Intra-assay reproducibility ^a				
Quality controls				
Midazolam	100	98.1 \pm 6.4	6.6	-1.9
	37.5	33.4 \pm 0.99	3.0	-10.0
	7.5	7.24 \pm 0.56	7.8	-3.5
Inter-assay reproducibility ^b				
Quality controls				
Midazolam	100	96.4 \pm 4.6	4.8	-3.6
	37.5	35.3 \pm 2.5	7.2	-5.9
	7.5	6.99 \pm 0.49	6.9	-6.7
Standards				
Midazolam	2	2.64 \pm 0.21	8.1	32.2
	5	4.37 \pm 0.44	9.9	-12.6
	10	9.75 \pm 1.1	11.8	-2.5
	25	23.6 \pm 2.3	9.6	-5.8
	50	51.8 \pm 3.9	7.6	3.6
	75	76.7 \pm 5.8	7.6	2.1
	125	125 \pm 7.7	6.2	0.2

^a Ten quality control samples per concentration

^b Five to ten quality control sample or two standards per day per concentration for 3 days

5.2.1.3 Selectivity and Stability for Midazolam

No endogenous interfering peaks were present in six sources of blank human plasma, and there was no carry-over evident in any of the blank reagent samples; therefore, the assay can be regarded as selective against possible matrix constituents.

5.2.1.4 Extraction Recovery

The mean extraction recovery of MDZ in plasma at 5 ng/mL was 152 ± 31.9 %. Recovery at 25 ng/mL for MDZ in plasma was 83.0 ± 1.32 %.

5.2.2 Validation of a HPLC-MS Assay for Detection of 1-Hydroxymidazolam in Urine

5.2.2.1 Chromatographic Separation for Urine Analysis of 1-Hydroxymidazolam

Representative chromatograms of plasma samples by SIM mass spectrometric detection are shown in **Figure 27**. Retention times for 1-OH MDZ and [$^2\text{H}_4$] –midazolam (internal standard, IS) were 6.79 and 6.97 minutes, respectively. The peaks of the compounds of interest were well separated and there was no interference from endogenous compounds during the elution window for the analytes and IS.

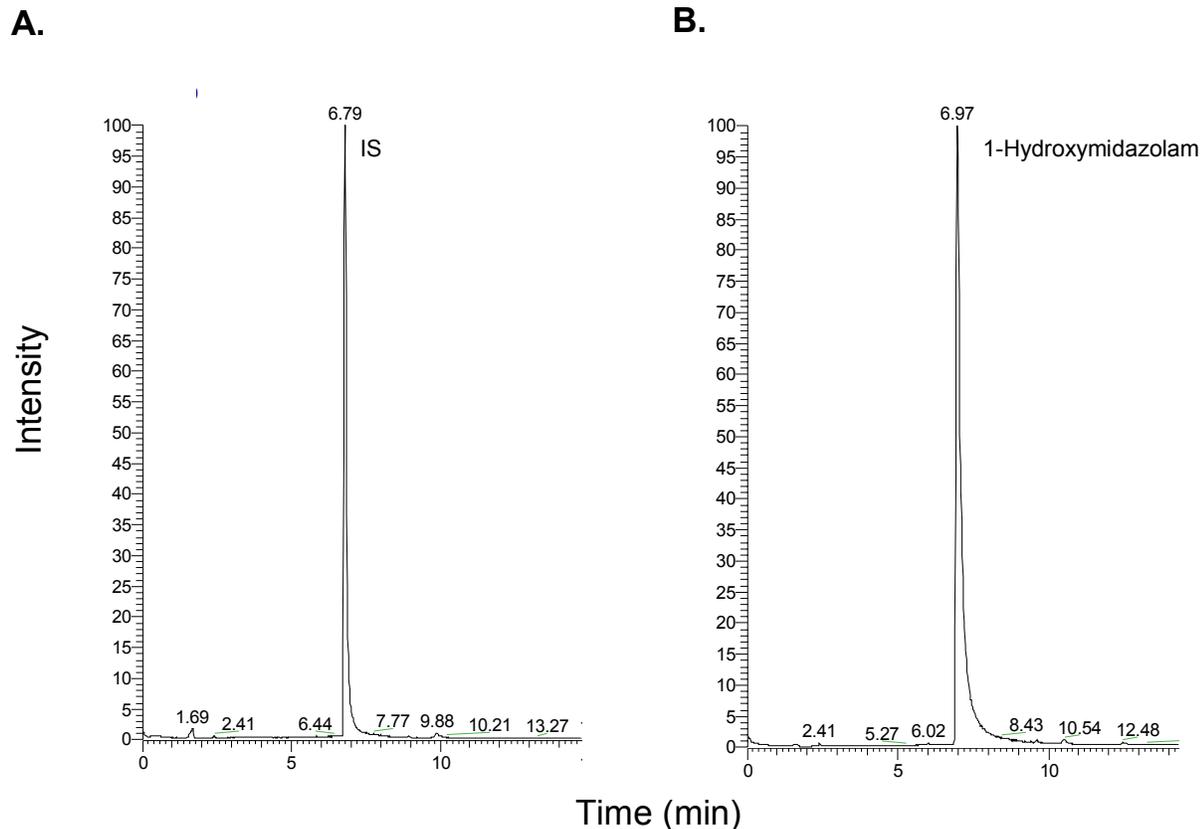


Figure 27: Representative SIM chromatograms of internal standard and 1-hydroxymidazolam

Internal standard (IS), midazolam D4 in Panel A, and 1- hydroxymidazolam in Panel B. The LOQ for 1-hydroxymidazolam is 12.5 ng/ml.

5.2.2.2 Linearity, Precision, and Calibration for 1-Hydroxymidazolam

Linear calibration curves were obtained for 1-OH MDZ over the concentration range of 125 to 2000 ng/mL. The mean regression equations (\pm SD) were as follows:

$$y = [0.005 (\pm 0.001)]x - 0.707 (\pm 0.503)$$

Correlation coefficients (r) were ≥ 0.974 for all standard curves. The lower limit of quantification (LOQ) for 1-OH MDZ (125 ng/mL or 2500 pg on column) demonstrated an

acceptable reproducibility (< 5%) and a signal-to-noise ratio of 12:1. The intra- and inter-day precision and accuracy were less than $\pm 15\%$ (Table 12).

Table 12: Intra- and inter-day precision and accuracy for 1-hydroxymidazolam

	Amount (ng/mL)		% R.S.D.	% Bias
	Added	Observed (mean \pm S.D.)		
Intra-assay reproducibility ^a				
Quality controls				
1-OH Midazolam	1750	1778 \pm 216	12.2	1.6
	650	597.9 \pm 42.5	7.1	-8.0
	200	221.2 \pm 9.88	4.5	10.6
Inter-assay reproducibility ^b				
Quality controls				
1-OH Midazolam	1750	1758 \pm 218	12.4	0.5
	650	594.9 \pm 39.6	6.7	-8.5
	200	220.3 \pm 7.92	3.6	10.2
Standards				
1-OH Midazolam	125	189.2 \pm 47.3	25.0	51.3
	250	282.9 \pm 33.9	12.0	13.2
	500	468.1 \pm 39.3	8.4	-6.4
	750	639.1 \pm 58.2	9.1	-14.8
	1000	828.6 \pm 184	22.2	-17.1
	1500	1526 \pm 98.9	6.5	1.8
	2000	1994 \pm 262	13.1	-0.3

^a Ten quality control samples per concentration

^b Five to ten quality control sample or two standards per day per concentration for 3 days

5.2.2.3 Selectivity and Stability

No endogenous interfering peaks were present in six sources of blank human urine, and there was no carry-over evident in any of the blank reagent samples; therefore, the assay can be regarded as selective against possible matrix constituents.

5.2.2.4 Extraction Recovery

The mean extraction recovery of 1-OH MDZ from urine at 250 ng/mL was 94 ± 2.7 %. Recovery at 1000 ng/mL for 1-OH MDZ in plasma was 70 ± 8.23 %.

5.2.3 Subject Recruitment

A total of 3 subjects were included in this study. All subjects were between 18 and 39 years old and no demographic differences were noted.

5.2.4 Core Temperature in Subjects

Figure 28 depicts the time-course of temperature after administration of warm saline and cold saline in each subject. Data depicts that the degree of cooling achieved $1.23 \pm 0.25^{\circ}\text{C}$. when subjects were administered cold saline. The average duration of hypothermia below 36.5°C during cold saline infusion was 80 ± 17.4 min. Subjects 1 and 3 had a greater duration of time below 36.5°C compared to Subject 2 (Subject 1: 88 min; Subject 3: 92 min; vs. Subject 2: 60 min).

5.2.5 Pharmacokinetics of Midazolam in Subjects

Midazolam pharmacokinetic parameters are presented in **Table 13**, and plasma concentration-time curves for each subject are shown in **Figure 29**. Subject 1 demonstrated a decrease in Cl_s during cold saline infusion (54.8 ml/min) compared to warm saline (76.2 ml/min) as shown in

Figure 30. Subject 3 also demonstrated a decrease in the Cl_S of MDZ when administered cold saline (117 ml/min) compare to warm saline (133 ml/min). The Cl_S of MDZ in Subject 2 was

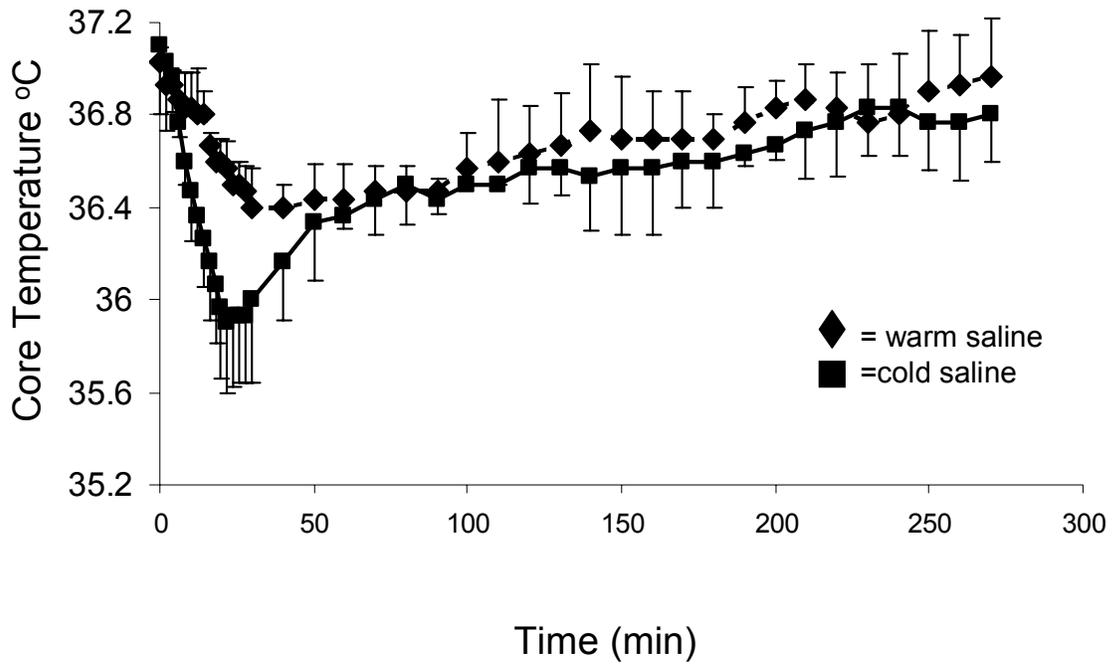
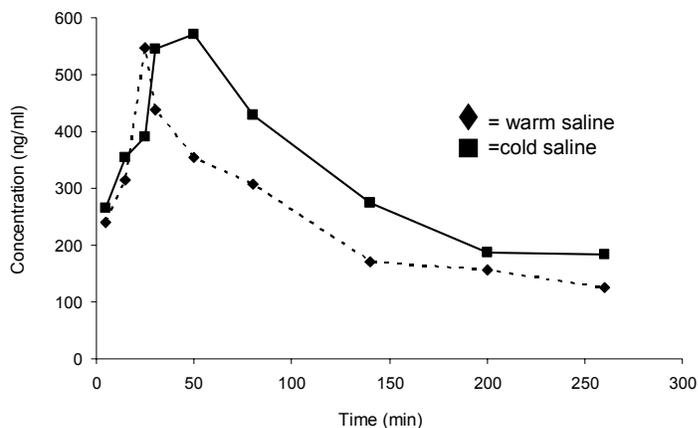
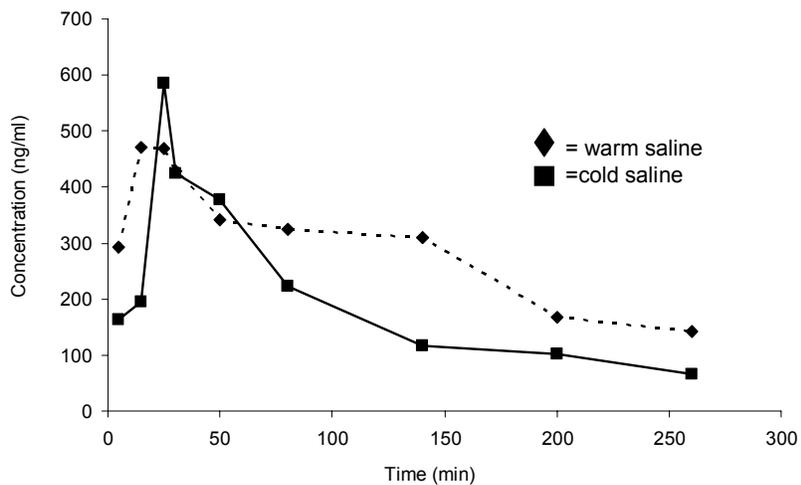


Figure 28: Core temperature during warm (◆) and cold saline infusion (■)

Subject 1



Subject 2



Subject 3

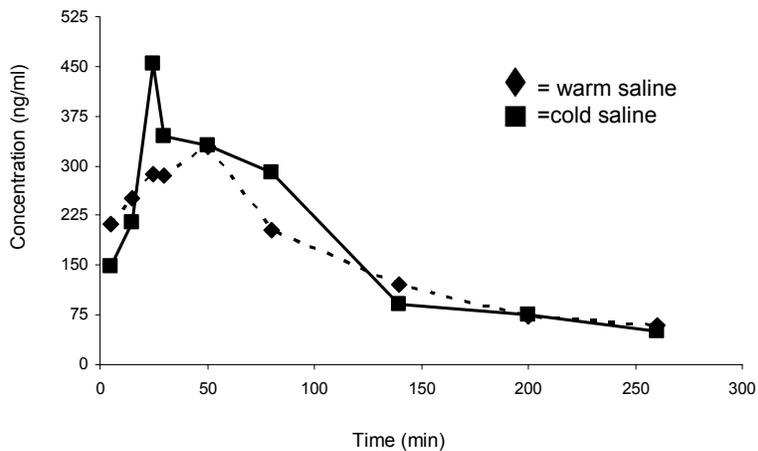


Figure 29: Plasma concentration-time curve for midazolam in subjects

increase during cold saline administration vs. warm saline (108.4 vs. 63.2 ml.min). When the data was combined, there was no statistically significant difference in the Cl_s of MDZ when subjects were warm (90.8 ± 37.1 ml/min) vs. cold (93.4 ± 33.7 ml/min). Both Subjects 1 and 3 demonstrated increases in the $AUC_{(0-\infty)}$ of MDZ when administered cold saline (Subject 1: 109,528.9 vs. 78,739.2 ng*min/ml, Subject 2: 51,303.1 vs. 45,127.5 ng*min/ml) vs. warm saline. Subject 2 showed a decrease in the $AUC_{(0-\infty)}$ of MDZ when administered cold saline (55,348.4 ng*min/ml) vs. warm saline (94,900.8 ng*min/ml). When the data was combined, there was no statistically significant difference in the $AUC_{(0-\infty)}$ of MDZ when subjects were warm ($73,920 \pm 26,716$ ng*min/ml) vs. cold ($72,059 \pm 32,511$ ng*min/ml). Additionally, there was a decrease in the V_{ss} when administered cold saline (13.1 ± 3.7) vs. warm saline (15.2 ± 2.8).

Table 13: Midazolam pharmacokinetic parameters

	C_{max} (ng/ml)	t_{max} (min)	$t_{1/2}$ (min)	AUC (ng*min/ml)	V_{ss} (L)	Cl_s (ml/min)	λ_z (min^{-1})
subject 1							
warm (37°C)	546.4	25	126.0	78739.2	14.2	76.2	0.0055
cold (4°C)	571.0	50	123.8	109528.9	10.4	54.8	0.0056
subject 2							
warm (37°C)	469.5	25	144.4	94900.8	13.0	63.2	0.0048
cold (4°C)	584.6	25	77.9	55348.4	11.6	108.4	0.0089
subject 3							
warm (37°C)	328.0	50	99.0	45127.5	18.3	133.0	0.007
cold (4°C)	454.6	25	144.4	51303.1	17.4	117.0	0.0048

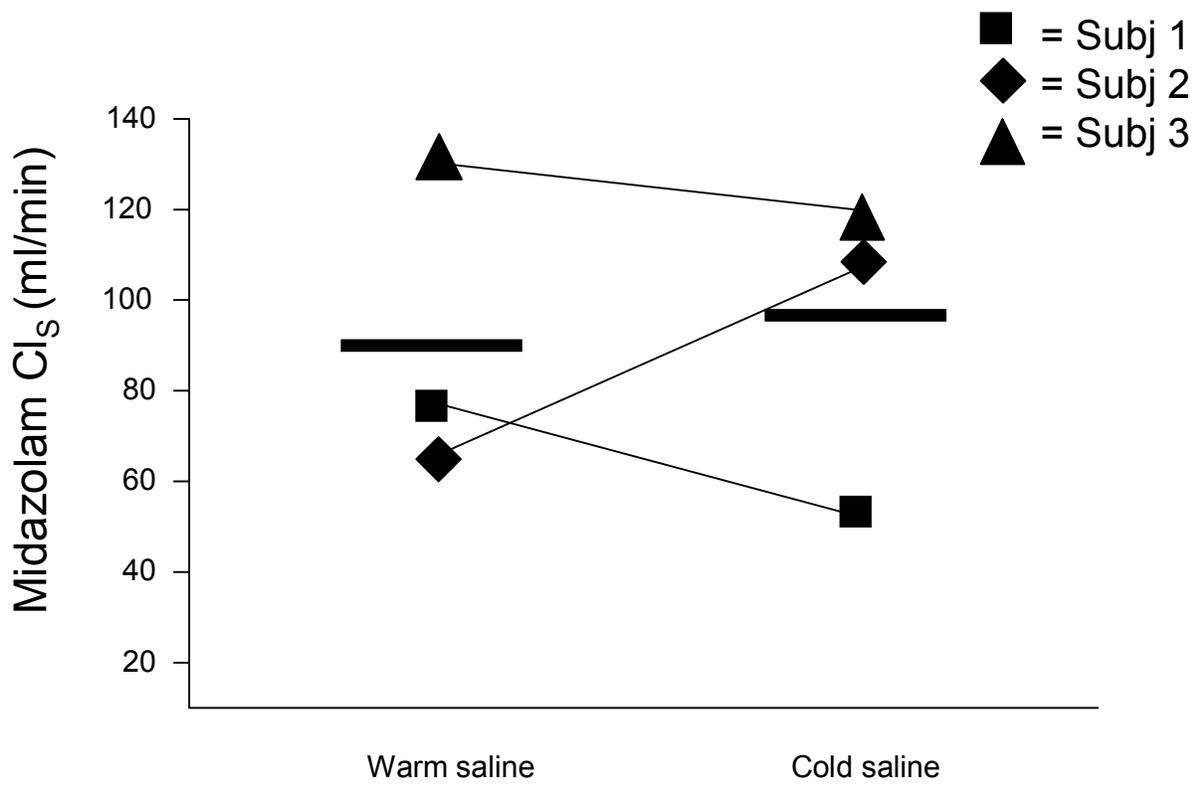


Figure 30: Systemic clearance (Cl_s) of midazolam in normal healthy subjects

Data depicts Cl_s during warm saline infusion and cold saline infusion. Mean value is represented by solid line. No statistical differences were observed.

5.3 DISCUSSION

This pilot study demonstrated three important findings. First, a rapid, sensitive, and selective method for determining MDZ in human plasma in normal-healthy males was developed using HPLC-MS methodology. Second, the administration of cold saline (4°C) to normal-healthy males decreases the core temperature by 1.23 ± 0.25 °C for 80 ± 17.4 min. Third, mild hypothermia does not alter the pharmacokinetics of the CYP3A4 probe substrate, MDZ, in normal healthy volunteers. These results represent the a preliminary clinical investigation to identify certain changes in drug metabolizing enzymes.

Development of a rapid, sensitive, and selective HPLC-MS assay for determining MDZ concentrations in human plasma and urine. MDZ is a short acting benzodiazepine commonly employed as a sedative. In humans, it is extensively metabolized by CYP3A4 and CYP3A5 isoforms to 1-OH MDZ and to a lesser extent 4-OH MDZ (Wandel, Bocker et al. 1994). MDZ is an established probe substrate for phenotyping CYP3A4 and CYP3A5 activity in human subjects (Lee, Chaves-Gnecco et al. 2002; Lepper, Baker et al. 2005). Thus, a rapid, sensitive, and selective analytical technique for the detection of MDZ is warranted. Although there have been multiple techniques reported, there have been a lack of HPLC-MS methods developed for determining plasma and urine concentrations of MDZ and 1-OH MDZ. The studies that have been reported typically use LC-MS-MS methods, which may not be available to all investigators. Additionally, other LC-MS techniques have been developed using high volumes of plasma (1 ml), which can be costly if multiple analyses are required. We have developed a rapid, sensitive and selective HPLC-MS method, using a smaller volume of plasma for determining MDZ and 1-OH MDZ concentrations. In addition, our method is the first HPLC-MS method for determining 1-OH MDZ in human urine.

Administration of ice-cold saline plus sedation decreases the body's core temperature.

One of the goals of this work was aimed at determining if the use of sedatives could prevent shivering and therefore, decrease and maintain hypothermia in normal healthy subjects over a 3 hr time period. The protocol used in this study achieved $\sim 1^{\circ}\text{C}$ of hypothermia and was maintained for ~ 70 minutes. Caldwell *et al* showed longer period of hypothermia in normal healthy volunteers due to the administration of neuromuscular blockers, such as vecuronium. Leslie *et al* achieved hypothermic temperatures (34°C) for ~ 104 minutes using surface cooling devices; however time to goal temperature was ~ 140 minutes. Our results indicate that our current protocol, hypothermia was not maintained for the entire 3 hrs. A solution to this problem would be the use of endovascular cooling for induction of hypothermia coupled with surface cooling devices to maintain hypothermia in order to achieve and maintain cooling at goal temperature. Furthermore, the use of other medications may inhibit the shivering response to a greater degree than the combination of MDZ and lorazepam. Future work is currently underway to optimize the protocol in order to provide longer duration of hypothermia in normal healthy subjects.

Preliminary analysis suggests that mild hypothermia does not alter MDZ pharmacokinetics. Our preliminary analysis does not demonstrate an impact of mild hypothermia on the pharmacokinetics of MDZ in normal healthy subjects. Specifically, we observed reductions in the Cl_s and V_{ss} and an increase in the AUC, in two of the 3 subjects. As outlined in Chapter 1, studies have shown that hypothermia decreases the Cl_s of drugs such as vecuronium, remifentanyl, and phenytoin (Caldwell, Heier *et al.* 2000; Iida, Nishi *et al.* 2001; Michelsen, Holford *et al.* 2001). Fukuoka *et al.* showed a 5-fold increase in plasma concentrations of MDZ when the core temperature of brain-injured patients was maintained

below 35°C (Fukuoka, Aibiki et al. 2004). This study cooled patients to 32 – 34°C for 3-4 days. Our results are consistent with this study demonstrating a decrease in the Cl_S of MDZ during hypothermia. Interestingly, our study shows that small decreases in core temperature (approximately 1°C of hypothermia for 75 min) alter the pharmacokinetics of MDZ in normal healthy males. Subject 2 demonstrated an increase in the Cl_S of MDZ and a decrease in the AUC; however the duration of hypothermia in this subject was much shorter than the other 2 subjects, which could possibly explain why these results are inconsistent with the other subjects. As mentioned earlier, MDZ is an established probe substrate with a low extraction ratio that is exclusively hepatically metabolized (Lee, Chaves-Gnecco et al. 2002; Lepper, Baker et al. 2005). Therefore, the Cl_H of MDZ approximates the Cl_S and is dependent on f_u and the Cl_{int} of CYP3A4. Results in Chapters 2 and 4 suggest that hypothermia alters the affinity of certain probe substrates for CYP450 isoforms. One potential explanation for the hypothermia-mediated decrease in the Cl_S of MDZ is that hypothermia decreases the affinity of MDZ for the CYP3A4 isoform. Collectively, these studies show that hypothermia impacts the disposition of certain hepatically eliminated drugs, more specifically by decreasing the Cl_S . Future goals of this work are to better control the duration of hypothermia and to increase the sample size.

Mild hypothermia decreased the V_{ss} of MDZ in normal healthy subjects. We showed a decrease in the V_{ss} during hypothermia. This is consistent with a study by Fukuoka et al, that demonstrated an increase in the V_d of MDZ when TBI patient's core temperature was below 35°C vs. when core temperature was normothermic (Fukuoka, Aibiki et al. 2004). The V_d is a term which relates the amount of drug in the body to the concentration in the blood or the plasma. The V_d calculated at steady state relates the concentration in the plasma with the amount in the body at one time point. A lower V_{ss} indicates that the drug is less distributed out of the

plasma. This is most likely due to the peripheral vasoconstriction due to the administration of ice-cold saline. Additional factors which could also affect the V_d are changes in protein binding, global blood perfusion to organs, diffusion across membranes, or drug-tissue binding. Previous studies have shown that hypothermia does not affect the protein binding of certain drugs and the other factors have never been investigated (Iida, Nishi et al. 2001; Tortorici, Kochanek et al. 2006).

5.4 CONCLUSIONS

We report that mild hypothermia does not alter the pharmacokinetic parameters of MDZ. This is the first systematic investigation into the effects of hypothermic therapy during cooling on CYP3A4-mediated metabolism in normal healthy volunteers. In addition, we also demonstrate that our cooling protocol induces mild hypothermia in normal healthy volunteers but does not maintain hypothermia over 3 hrs. Future studies are needed to assess the effects of hypothermia during cooling on other CYP450 isoforms as well as other enzymes which are involved in the metabolism of clinically relevant drugs. Although this is a pilot study, clinical trials are needed in order to demonstrate if these effects are present in a larger number of subjects. However, these data imply that clinically significant hypothermia-drug interactions are a likely factor to consider in the therapeutic course of these patients.

6.0 CHAPTER 6

Conclusions and Future Directions

The purpose of this research was to examine both the effects of therapeutic hypothermia on CYP450-mediated metabolism, during cooling and after re-warming. In order to begin to systematically evaluate these effects, a CA rat model was utilized to examine these effects on CYP2E1 and CYP3A2 metabolism. In addition, human liver microsomes were then utilized to examine the effects of mild and moderate hypothermia on CYP2E1 and CYP3A4 enzyme kinetics. Furthermore, this analysis was also used to predict temperature-mediated changes in patients treated with therapeutic hypothermia. Lastly, a pilot study was conducted to investigate the effects of mild hypothermia on CYP3A4 activity by analyzing the pharmacokinetics of MDZ in normal-healthy volunteers treated with ice-cold saline.

Results from Chapter 2 suggest that the application of moderate hypothermia in a CA rat model dramatically alters the pharmacokinetics of the CYP2E1 probe drug, CZN. Interestingly, we observed a ~2-fold reduction in the Cl_s of CZN. A potential mechanism behind these temperature-mediated changes was a decrease in the affinity of CYP2E1 for CZN. From this *in vitro* analysis, we accurately predicted changes in the *in vivo* Cl_s of CZN in a rat. This is the first systematic investigation into the acute effects of hypothermic therapy on CYP2E1-mediated metabolism and the first mechanistic insight into hypothermia induced reductions in CZN clearance.

Chapter 3 extended the analysis of the work performed in Chapter 2, investigating the post re-warming effects in the acute and sub-acute phases after CA injury on CYP3A2 and CYP2E1-mediated metabolism. This study demonstrated three novel findings. First, there was an increase in IL-6 plasma concentrations early after CA. IL-6 is known to decrease *CYP3A2* and *CYP2E1* expression. Second, CA produced a significant decrease in the hepatic activity of

CYP3A2 and CYP2E1 in rats, in the sub-acute phase, 24 hrs after injury compared to Control rats. Third, moderate hypothermia applied to CA rats, prevented both the down-regulation of CYP3A2 and CYP2E1-metabolism and blocked the early increase in IL-6 seen after CA Normothermia.

In order to translate the results from Chapter 2 from rat to human, we performed an enzyme kinetic analysis in human liver microsomes at both mild and moderate hypothermic temperatures. Mild and moderate hypothermia decreased the Cl_{int} of CYP2E1-mediated metabolism of CZN however, did not affect Cl_{intmax} of CYP3A4-mediated metabolism of TST in pooled human hepatic microsomes. These temperature-induced alterations were due to decreases in the V_{max} of both CYP3A4 and CYP2E1 and an increase in the K_m of the CYP2E1 enzyme. Individual human liver microsomes incubated at both mild and moderate hypothermic temperatures demonstrated a rank order decrease in the Cl_{int} of CYP2E1 and CYP3A4. These data provide insight into temperature-induced alterations on CYP450-mediated in human liver, specifically identifying that CYP isoforms may be differentially affected by therapeutic hypothermia.

Lastly, a pilot investigation of the effects of mild hypothermia on the pharmacokinetics of MDZ suggests that CYP3A4 activity is not altered in normal healthy volunteers with the current model of cooling we are utilizing. We did observe specific changes in individual subjects including, a decrease in the Cl_s of MDZ in two of 3 subjects compared to when subjects were treated with warm saline. In addition, each subject demonstrated a decrease in the V_{ss} of MDZ during ice-cold saline infusion compared to warm saline.

This body of work provides the foundation for future preclinical studies and clinical studies. One of the major conclusions of this work is that mild and moderate hypothermia alters

the enzyme kinetics of CYP450 isoforms. Additional studies investigating the effects of hypothermia on multiple enzyme kinetic models including models of atypical enzyme kinetics are warranted. This will provide mechanistic insight into the effect of mild to moderate cooling on CYP450 enzyme functionality. Additionally, it will be important to investigate the effects of hypothermia on other drug metabolizing enzymes such as UDP-glucuronosyltransferase as well as drug transporters, such as p-glycoprotein.

Another future direction of this work includes the further investigation of the effects of cardiac arrest with and without hypothermia on the effects of pro-inflammatory cytokines and the regulation of CYP450s. First, further investigation into the role of IL-6 could be analyzed using an IL-6 knockout mouse model. This study would include a control group without cardiac arrest or hypothermia, control group receiving cardiac arrest, IL-6 knockout receiving cardiac arrest, and IL-6 knockout receiving cardiac arrest plus hypothermia. The use of this model would provide insight into the role of IL-6 on the regulation of CYP450 isoforms after cardiac arrest. The investigation into the role of other pro-inflammatory cytokines (IL-1 β and TNF- α) and nuclear transcription factors (AP-1 and C/EBP beta) needs to be investigated as inflammation is regulated via multiple pathways.

These preclinical studies are important to provide direction as we develop future clinical studies identifying the effects of hypothermia on drug metabolism. The findings of our pilot analysis need to be confirmed by enrolling a greater number of subjects and utilizing a CYP450 cocktail approach in order to assess multiple CYP isoforms in each subject. In addition, the development of a hypothermia model in normal healthy volunteers which would maintain mild hypothermia over a longer period of time is warranted to provide a more controlled study. The

use of a pharmacodynamic measure of sedation during hypothermia would provide insight into the effects of cooling on drug response and correlate this to the pharmacokinetic alterations.

These data then can be used to develop a multi-center clinical trial investigating the effects of therapeutic hypothermia on CYP450-mediated metabolism in CA patients. Second, as outlined in Chapter 1, the effects of hypothermia on drug disposition are a complex interplay between the effects during cooling, and the effects of hypothermia after re-warming) and the effects on drug response. The effects of hypothermia on both pharmacokinetic and pharmacodynamics would As this work has begun to investigate these effects of hypothermia on drug metabolism, future work is needed to further elucidate these changes and examine hypothermia-mediated changes in drug response.

In conclusion, the results of the work performed throughout this dissertation could have important clinical implications, since reductions in metabolism could lead to drug toxicity and adverse events in patients treated with therapeutic hypothermia. In order to begin to provide dosing recommendation during hypothermia therapy, more research is warranted in order to optimize patient care.

APPENDICES

APPENDIX A: Data From All Experimental Analyses

A.1 STUDY 1: THERAPEUTIC HYPOTHERMIA –INDUCED PHARMACOKINETIC ALTERATIONS ON CYP2E1 CHLORZOXAZONE-MEDIATED METABOLISM IN A CARDIAC ARREST RAT MODEL

A.1.1 Effects of Moderate Hypothermia on Chlorzoxazone Plasma Concentrations ($\mu\text{g/mL}$) After Cardiac Arrest

CA Normothermia Animals						
Time (minutes)	1	2	3	4	5	6
10	17.8	15.4	14.6	16.5	18.6	18.5
20	12.8	13.6	19.8	17.4	20.9	20.4
30	14.5	13.3	18.9	26.8	24.6	20.2
60	13.8	14.9	16.7	18.5	22.8	17.0
90	17.9	12.8	11.3	17.1	17.1	23.9
120	13.2	10.3	12.7	14.5	14.0	20.8
150	17.4	11.6	9.2	15.9	13.1	16.5
165	20.4	8.5	10.0	18.1	13.0	15.4
CA Hypothermia Animals						
	1	2	3	4	5	6
10	18.3	16.2	18.3	19.9	21.3	13.9
20	21.4	16.6	15.3	18.9	21.8	9.8
30	18.1	12.8	15.2	15.3	19.1	12.8
60	11.6	8.6	10.4	9.8	16.2	10.9
90	10.7	8.0	8.6	8.4	15.9	10.2
120	8.4	10.3	7.9	6.6	14.9	8.2
150	7.3	9.9	10.0	5.8	13.8	8.0
165	6.4	8.0	8.2	7.9	13.1	5.2

A.1.2 Urine Analysis of 6-OH Chlorzoxazone of Cardiac Arrest Animals

	Concentration (ug/m	Volume(mL)	Amount (ug)	of Excretion (ug/min)
CA Normothermia Animals				
1	552.3	0.7	386.6	2.3
2	201.4	0.4	80.56	0.5
3	330.6	0.6	198.3	1.2
4	292.5	0.75	219.4	1.3
5	104.9	0.9	94.39	0.6
6	107.9	0.6	64.76	0.4
CA Hypothermia Animals				
1	23.36	0.7	16.35	0.10
2	95.86	0.2	19.17	0.12
3	139.4	0.8	111.5	0.68
4	18.53	0.4	7.410	0.04
5	99.52	0.7	69.66	0.42
6	58.02	0.5	29.01	0.18

A.1.3 Effects of Moderate Hypothermia on Protein Binding of Chlorzoxazone

	Total (ug/mL)	Bound (ug/mL)	Unbound (ug/mL)	Fraction Unbound	% Bound
37°C Samples					
1	10.93	10.12	0.58	0.05	94.67
2	14.44	12.07	0.64	0.04	95.55
3	15.91	12.95	0.75	0.05	95.30
4	13.04	10.80	0.50	0.04	96.15
30°C Samples					
1	10.12	8.86	0.37	0.04	96.32
2	11.96	10.96	0.50	0.04	95.80
3	16.21	13.42	0.71	0.04	95.64
4	13.18	12.33	0.61	0.05	95.35

A.1.4 Formation Rates of 6-OH Chlorzoxazone (nmol/mg/min) in Michaelis-Menten Enzyme Kinetic Analysis

37°C Samples				
CZN Conc. (umol)	1	2	3	4
500	1.27	1.25	1.67	1.63
400	1.52	1.15	1.54	1.51
300	1.21	1.05	1.30	1.38
200	0.97	0.96	0.99	1.27
100	0.58	0.55	0.77	0.70
75	0.39	0.36	0.57	0.56
50	0.28	0.18	0.48	0.40

30°C Samples				
CZN Conc. (umol)	1	2	3	4
500	0.99	1.57	1.02	1.53
400	0.95	1.45	1.17	1.29
300	0.85	1.35	0.86	0.96
200	0.67	0.94	0.67	0.76
100	0.38	0.46	0.37	0.38
75	0.37	0.28	0.30	0.35
50	0.23	0.14	0.24	0.20

A.2 STUDY 2: MODERATE HYPOTHERMIA PREVENTS CARDIAC ARREST-MEDIATED CYP3A2 AND CYP2E1 SUPPRESSION AND INTERLEUKIN-6 INDUCTION

A.2.1 Formation Rates of 6-OH Chlorzoxazone (nmol/mg/min) in Cardiac Arrest Rats 24-hours After Injury

Control		CA Normothermia		CA Hypothermia	
N2	0.88	CA 1	0.58	CA H1	0.40
N3	0.73	CA 2	0.44	CA H2	1.39
N4	0.64	CA 3	0.30	CA H3	1.21
N5	0.85	CA 4	0.54	CA H4	0.86
N6	1.02	CA 5	0.50	CA H5	0.70
N7	0.98	CA 6	0.47	CA H6	1.03
Average	0.85	Average	0.47	Average	0.93
St.Dev.	0.14	St.Dev.	0.10	St.Dev.	0.36

A.2.2 Formation Rates of 6 β -OH Testosterone (pmol/mg/min) in Cardiac Arrest Rats 24-hours After Injury

Control		CA Normothermia		CA Hypothermia	
N2	1026.0	CA 1	1103.9	CA H1	3684.8
N3	1817.1	CA 2	294.0	CA H2	2172.9
N4	1183.0	CA 3	504.8	CA H3	1861.2
N5	1724.7	CA 4	177.1	CA H4	1172.9
N6	1527.9	CA 5	947.0	CA H5	3634.5
N7	2626.6	CA 6	605.2	CA H6	5396.9
Average	1650.9	Average	605.3	Average	2987.2
St.Dev.	567.4	St.Dev.	362.2	St.Dev.	1545.0

A.2.3 Formation Rates of 6-OH Chlorzoxazone (nmol/mg/min) in Cardiac Arrest Rats 5-hours After Injury

Control		CA Normothermia		CA Hypothermia	
N1	1.19	CA 1	1.96	CA H1	2.05
N2	1.61	CA 2	1.61	CA H2	1.29
N3	1.16	CA 3	1.75	CA H3	2.21
N4	1.99	CA 4	1.79	CA H4	1.53
N5	1.79	CA 5	1.07	CA H5	1.47
N6	1.79	CA 6	1.55	CA H6	1.81
Average	1.59	Average	1.62	Average	1.73
St.Dev.	0.34	St.Dev.	0.31	St.Dev.	0.36

A.2.4 Formation Rates of 6 β -OH Testosterone (pmol/mg/min) in Cardiac Arrest Rats 5-hours After Injury

Control		CA Normothermia		CA Hypothermia	
N2	2012.6	CA 1	2741.2	CA H1	1867.8
N3	2660.8	CA 2	2588.3	CA H2	2521.2
N4	2153.7	CA 3	2361.6	CA H3	3510.6
N5	3166.7	CA 4	2496.2	CA H4	2186.7
N6	2582.9	CA 5	1946.7	CA H5	1865.3
N7	1756.2	CA 6	2112.1	CA H6	3171.5
Average	2388.8	Average	2374.4	Average	2520.5
St.Dev.	512.6	St.Dev.	299.0	St.Dev.	688.6

A.2.5 Interleukin-6 Plasma Concentrations (pg/ml) in Cardiac Arrest Rats After Injury

Time (min)							
Control		1	2	3	4	5	6
	60	42.8	81.2	158.2	6.6	125.7	0.0
	70	10.9	0.0	103.8	7.0	90.4	0.0
	90	31.1	32.4	49.2	9.6	22.5	0.0
	120	56.5	23.9	30.4	0.0	0.0	0.0
	180	21.9	16.1	28.5	28.5	0.0	0.0
	210	12.2	25.9	19.1	47.9	0.0	0.0
	240	53.9	43.4	35.1	39.9	0.0	45.0
	270	34.3	33.7	77.1	53.1	29.0	27.3
	285	16.7	0.0	0.9	89.6	16.1	37.8
CA Normothermia		1	2	3	4	5	6
	60	958	812	386	566	985	877
	70	543	873	412	385	2233	826
	90	714	569	262	161	651	518
	120	298	465	201	46	294	249
	180	204	242	180	11	160	215
	210	341	350	326	40	164	399
	240	129	495	204	30	336	314
	270	33	271	170	25	254	340
	285	109	216	180	36	211	297
CA Hypothermia		1	2	3	4	5	6
	60	121.4	35.1	29.3	9.9	15.8	91.1
	70	83.8	41.7	6.1	18.4	8.7	80.2
	90	45.6	43.9	6.6	16.8	9.5	61.3
	120	27.8	35.5	13.6	28.4	8.3	74.0
	180	19.7	46.6	12.7	25.8	9.5	71.2
	210	48.3	36.0	21.0	38.4	12.3	47.2
	240	32.0	22.3	30.7	47.6	7.4	52.3
	270	35.2	0.0	43.2	83.0	10.4	57.5
	285	14.4	0.0	32.4	22.3	8.1	65.1

A.2.6 Nitrate Plasma Concentrations (μmol) in Cardiac Arrest Rats After Injury

		Time (min)					
Control		1	2	3	4	5	6
	60	4.8	6.9	17.5	13.2	30.3	0.0
	70	5.2	7.0	18.2	11.2	15.9	9.1
	90	5.9	6.2	23.2	14.8	11.6	11.5
	120	5.5	7.7	24.1	11.2	13.0	0.0
	180	7.5	8.3	19.9	11.8	9.9	0.0
CA Normothermia		1	2	3	4	5	6
	60	6.3	0.0	12.4	12.6	15.2	19.0
	70	8.9	28.9	13.8	16.8	11.4	13.6
	90	8.3	25.7	10.6	8.4	14.2	17.2
	120	14.2	30.4	16.5	20.5	14.9	23.3
	180	15.4	37.3	13.7	15.8	29.7	0.0
CA Hypothermia		1	2	3	4	5	6
	60	12.1	11.3	11.9	0.0	15.8	0.0
	70	0.0	10.8	11.0	15.1	8.7	11.4
	90	0.0	0.0	17.1	16.3	9.5	0.0
	120	0.0	13.2	12.2	20.1	8.3	13.0
	180	16.1	15.7	12.5	15.7	9.5	17.7

A.2.7 Nitrite Plasma Concentrations (μmol) in Cardiac Arrest Rats After Injury

		Time (min)					
Control		1	2	3	4	5	6
	60	0.03	0.00	4.7	4.1	11.4	0.0
	70	0.03	0.00	3.5	5.8	9.2	7.4
	90	0.00	0.00	3.6	5.2	8.2	9.7
	120	0.05	0.00	4.2	3.9	8.7	0.0
	180	0.00	0.00	3.8	4.3	6.0	0.0
CA Normothermia		1	2	3	4	5	6
	60	0.0	0.0	6.3	8.8	10.6	11.8
	70	0.1	7.8	4.4	14.2	7.8	8.0
	90	0.0	6.1	10.4	6.3	8.3	8.0
	120	0.1	5.2	6.5	14.9	8.9	9.4
	180	0.0	10.0	4.0	10.0	13.0	0.0
CA Hypothermia		1	2	3	4	5	6
	60	3.9	5.7	4.3	0.0	0.0	0.0
	70	3.6	0.0	3.8	18.2	14.5	14.5
	90	2.9	0.0	0.0	13.8	0.0	0.0
	120	4.2	0.0	5.7	13.9	15.9	15.9
	180	2.6	0.0	7.7	16.1	0.0	0.0

A.3 STUDY 3: MILD AND MODERATE HYPOTHERMIA ALTERS CYP2E1 AND CYP3A4 ENZYME KINETICS IN HUMAN HEPATIC MICROSOMES

A.3.1 Effect of Mild and Moderate Hypothermia on CYP2E1 Michaelis Enzyme Kinetics: Formation Rates of 6-OH Chlorzoxazone (nmol/mg/min) in Pooled Human Hepatic Microsomes

37°C Samples	1	2	3	4	5	6	Average	St.Dev.
CZN Conc. (umol)								
500	1.94	1.80	2.06	1.94	1.74	2.40	1.98	0.23
400	1.97	1.78	2.18	1.94	1.69	2.37	1.99	0.25
300	1.81	1.84	2.11	1.95	1.89	2.25	1.98	0.17
200	1.96	2.01	2.07	1.87	1.75	1.90	1.93	0.11
100	1.65	1.23	1.71	1.33	1.31	1.46	1.45	0.20
75	1.12	1.13	1.28	0.92	0.92	0.77	1.02	0.19
50	0.80	1.06	0.93	0.72	0.69	0.63	0.81	0.16
33°C Samples	1	2	3	4	5	6	Average	St.Dev.
CZN Conc. (umol)								
500	1.11	1.16	1.08	1.19	1.16	1.24	1.16	0.06
400	1.00	1.25	1.13	1.21	1.34	0.94	1.14	0.15
300	0.89	1.10	1.07	0.94	1.10	0.96	1.01	0.09
200	0.76	1.12	1.11	0.95	1.14	1.00	1.01	0.15
100	0.85	0.68	0.74	0.72	0.65	0.76	0.73	0.07
75	0.64	0.70	0.59	0.79	0.69	0.61	0.67	0.07
50	0.50	0.43	0.38	0.43	0.48	0.54	0.46	0.06
30°C Samples	1	2	3	4	5	6	Average	St.Dev.
CZN Conc. (umol)								
500	1.06	0.91	0.87	1.55	1.55	1.57	1.25	0.34
400	0.85	0.95	0.97	1.42	1.92	1.51	1.27	0.42
300	0.87	0.83	0.98	1.26	1.54	1.23	1.12	0.27
200	0.75	0.94	0.68	1.22	1.31	1.09	1.00	0.25
100	0.50	0.50	0.61	0.89	1.04	0.82	0.73	0.22
75	0.46	0.36	0.49	0.85	0.66	0.60	0.57	0.17
50	0.05	0.25	0.39	0.72	0.61	0.37	0.40	0.24

**A.3.2 Effect of Mild and Moderate Hypothermia on CYP3A4 Michaelis Enzyme Kinetics:
Formation Rates of 6 β -OH Testosterone ($\mu\text{mol}/\text{mg}/\text{min}$) in Pooled Human Hepatic
Microsomes**

37°C Samples	1	2	3	4	5	6	Average	St.Dev.
TST Conc. (μmol)								
250	9732.7	7074.3	8376.7	8212.1	7722.6	8884.5	8223.7	984.0
150	9473.8	9970.5	10380.4	7725.6	7295.6	7440.5	8969.2	1378.1
100	5683.3	6914.4	6454.7	8028.8	6251.4	5289.7	6666.5	880.5
75	7880.2	7436.2	6690.5	6187.4	6262.2	6663.8	6891.3	742.7
50	5804.3	5033.3	4169.8	5233.4	5423.9	3051.9	5133.0	608.9
25	2701.7	3945.7	4297.8	2187.0	3003.2	2727.9	3227.1	876.2
12.5	1173.0	991.2	945.9	2010.5	1621.6	1796.5	1348.4	456.4
6.25	414.8	385.2	418.7	434.1	320.9	244.8	394.7	44.9
33°C Samples	1	2	3	4	5	6	Average	St.Dev.
TST Conc. (μmol)								
250	3933.7	4071.5	3322.7	3711.9	3940.1	3727.3	3796.0	294.4
150	3842.2	3763.2	3932.4	3944.0	3570.7	3389.1	3810.5	152.8
100	4279.4	3077.6	3312.0	3457.3	3471.4	3226.9	3519.5	453.3
75	3454.7	3580.0	3354.7	3500.6	3447.6	3530.3	3467.5	82.2
50	2912.1	3429.1	2957.5	3012.9	2442.3	2854.3	2950.8	351.0
25	2090.5	2799.1	2300.5	2062.6	1965.2	2035.9	2243.6	333.8
12.5	992.6	1317.5	1394.7	1052.1	996.7	938.8	1150.7	190.9
6.25	381.6	299.1	547.5	346.0	463.5	383.3	407.5	98.7
30°C Samples	1	2	3	4	5	6	Average	St.Dev.
TST Conc. (μmol)								
250	4071.2	4071.5	3322.7	3711.9	3940.1	3727.3	3807.4	285.5
150	4286.1	3763.2	3932.4	3944.0	3570.7	3389.1	3814.3	315.0
100	3664.5	3077.6	3312.0	3457.3	3471.4	3226.9	3368.3	206.9
75	3815.3	3580.0	3354.7	3500.6	3447.6	3530.3	3538.1	156.1
50	3105.1	3429.1	2957.5	3012.9	2442.3	2854.3	2966.9	323.4
25	2006.3	2799.1	2300.5	2062.6	1965.2	2035.9	2194.9	318.6
12.5	1197.4	1317.5	1394.7	1052.1	996.7	938.8	1149.5	183.2
6.25	412.7	299.1	547.5	346.0	463.5	383.3	408.7	88.2

A.3.3 Effect of Mild and Moderate Hypothermia on Formation Rates of 6-OH Chlorzoxazone (nmol/mg/min) in Individual Human Hepatic Microsomes

37°C Samples	1	2	3	4
CZN Conc. (umol)				
500	0.95	1.30	1.14	0.23
400	0.92	1.64	1.34	0.19
300	0.78	1.43	1.70	0.16
200	0.71	1.48	1.14	0.15
100	0.75	1.19	0.93	0.14
75	0.48	1.01	0.63	0.13
50	0.40	0.70	0.51	0.10
33°C Samples	1	2	3	4
CZN Conc. (umol)				
500	0.82	0.99	1.30	0.24
400	0.92	0.94	1.64	0.26
300	0.82	1.03	1.43	0.27
200	0.84	0.84	1.30	0.16
100	0.70	0.81	1.19	0.12
75	0.52	0.48	0.58	0.14
50	0.22	0.38	0.55	0.11
30°C Samples	1	2	3	4
CZN Conc. (umol)				
500	0.59	0.60	1.32	0.20
400	0.50	0.58	1.36	0.19
300	0.57	0.67	1.23	0.17
200	0.41	0.47	1.00	0.12
100	0.37	0.42	0.68	0.10
75	0.22	0.31	0.63	0.09
50	0.23	0.29	0.49	0.08

A.3.4 Effect of Mild and Moderate Hypothermia on Formation Rates of 6 β -OH Testosterone (pmol/mg/min) in Individual Human Hepatic Microsomes

37°C Samples	1	2	3	4
TST Conc. (umol)				
250	3386.6	8167.9	770.0	14130.0
150	3249.1	8744.5	1085.6	11026.5
100	3754.5	7475.8	1200.7	10650.0
75	3916.2	7238.8	700.2	10219.0
50	3566.6	6661.6	602.8	10577.2
25	3093.6	5293.8	546.3	12194.5
12.5	2048.9	3728.8	347.3	9635.0
6.25	908.9	2282.5	188.4	3513.1
33°C Samples	1	2	3	4
TST Conc. (umol)				
250	3693.2	6608.0	708.9	9809.9
150	3470.7	12169.4	965.0	11954.2
100	3308.1	5445.7	1024.5	10150.1
75	3533.4	7499.3	571.6	9163.5
50	3452.0	7127.8	499.5	8695.9
25	2406.6	5143.3	435.3	7390.2
12.5	1932.5	2750.0	285.0	4878.5
6.25	745.5	1811.1	172.8	1326.5
30°C Samples	1	2	3	4
TST Conc. (umol)				
250	3828.8	6001.4	683.0	8479.1
150	3990.9	6078.4	686.0	8724.5
100	3404.4	6125.3	718.0	9374.9
75	2855.5	5629.6	494.2	8902.2
50	2888.2	5299.4	455.2	8322.6
25	2475.5	3716.2	346.8	7432.3
12.5	830.0	1570.1	275.8	4755.2
6.25	950.3	874.9	192.9	2692.4

A.4 STUDY 4: THE EFFECTS OF MILD HYPOTHERMIA ON MIDAZOLAM PHARMACOKINETICS IN NORMAL HEALTHY SUBJECTS: A PILOT STUDY

A.4.1 Midazolam Plasma Concentrations in Subjects

Midazolam Concentrations

Subject 1	Warm(ng/ml)	Cold (g/ml)	Subject 2	Warm(ng/ml)	Cold (g/ml)	Subject 3	Warm(ng/ml)	Cold (ng/ml)	
	5	240	266	5	293	164	5	212	148
	15	315	355	15	472	196	15	250	215
	25	546	391	25	470	585	25	288	455
	30	438	545	30	429	425	30	285	346
	50	354	571	50	341	378	50	328	332
	80	307	430	80	325	222	80	204	290
	140	170	274	140	310	117	140	121	91
	200	156	188	200	168	103	200	73	75
	260	125	184	260	143	66	260	60	51

APPENDIX B: Study Approval, Protocol, and Consent Forms

B.1 IRB PROTOCOL

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Protocol Title:

Induction and maintenance of therapeutic hypothermia and associated pharmacokinetics in healthy subjects

1.0 SPECIFIC AIMS & OBJECTIVES

Specific Aim 1: Examine the administration of 4°C and 37°C saline in conjunction with benzodiazepine sedation or benzodiazepine sedation and magnesium sulfate infusion in an effort to blunt the shivering response and discomfort associated with cold saline infusion.

Specific Aim 2: Determine the pharmacokinetics of midazolam in normothermic and hypothermic subjects in order to assess CYP3A4 activity

2.0 BACKGROUND AND SIGNIFICANCE

Mild hypothermia (34-35°C) has been shown to improve neurological outcome after cardiac arrest.^{1,2} Animal studies have shown the therapeutic benefit may be optimized by earlier initiation following resuscitation.³ Surface cooling is non invasive and potentially the safest mode for inducing mild hypothermia. However, this technique has a limited ability to quickly bring the patient to the target temperature. Additionally, surface cooling requires substantial resources such as large quantities of ice, chemical icepacks, or powered cooling blankets. Endovascular cooling delivers chilled saline directly to the body core and bypasses the need to cool the shell and skeletal muscle.⁴ Since it is less costly and resource intensive, this mode of cooling could be initiated by paramedics in the prehospital setting.

Mild hypothermia has also been investigated as a therapeutic agent for stroke and myocardial infarction.^{5,6} As with cardiac arrest, the optimal core temperature is believed to be 34-35°C. However, unlike the post resuscitation patient, stroke patients will defend their core temperature and the shivering response may prevent the attainment of a therapeutic temperature. Significant cooling can be achieved under general anesthesia but this procedure is not available to paramedics.⁷ To initiate therapeutic hypothermia at the earliest opportunity it will be necessary to identify a regimen that is comfortable for the patient and suppresses or dampens the shivering response without jeopardizing patient safety. Additionally, this regimen must maintain therapeutic hypothermia induced in the prehospital setting until the patient can receive reperfusion therapy in the hospital.

Various drugs are available that may suppress the shivering response and yet still be safe to administer to awake patients during acute illness such as myocardial infarction or stroke. Meperidine has been used but has toxic metabolites. Other possibilities include narcotics, benzodiazepines, and buspirone. In addition, hypothermia has been shown to dramatically alter multiple drug concentrations, including benzodiazepines.⁸ In order to optimize this protocol and provide maximum comfort to the patient, it is crucial to understand the effect of cooling on the disposition and metabolism of drugs which are commonly employed by paramedics in the prehospital setting.

3.0 RESEARCH DESIGN AND METHODS

3.1 Drug/Device Information. This study does not utilize experimental drugs or devices. Benzodiazepine administration, normal saline administration, temperature monitoring are being provided as an on label indication.

3.2 Study Protocol

This protocol will examine the administration of midazolam to induce mild sedation followed by and lorazepam to maintain sedation during cold saline infusion to determine if a core temperature of 34-35°C can be achieved in healthy volunteers aged 18 – 35 years after mild sedation. We are utilizing two benzodiazepines to induce and maintain sedation to allow this protocol to determine the effects of mild hypothermia on the disposition of midazolam (Figure 1). Intensive monitoring will be performed to test the safety of this procedure in a patient population. A total of five visits will be required to complete the study. One visit will be required for the screening and four additional visits for the experimental protocols.

A study physician will perform a history and physical exam on each subject to assess for the presence of medical conditions. The screening will take place in the department of emergency medicine offices on a visit separate from the experimental protocol and is expected to take approximately 60 minutes. Special focus will be placed on a history of early cardiac death in family members or any other signs that an underlying cardiac anomaly may be present. Body composition will be assessed by measuring skinfolds at three sites. Blood samples will be collected for sodium, potassium, TSH, SGOT, SGPT, alkaline phosphatase, bilirubin, BUN, creatinine, and a complete blood count to exclude undiagnosed liver, hematologic, and metabolic disease. A maximum of 30 cc (2 tablespoon) of blood will be collected. Finally, a standard 12-lead EKG will be obtained and interpreted by a study physician to assess for any other apparent cardiac pathology. Normal lab values used by the University of Pittsburgh Medical Center Presbyterian Hospital lab will be accepted as the normal range. In the event a lab value is outside the normal range, but not clinically significant in the study physicians opinion, the subject will be allowed to repeat the lab test once. Two lab tests outside the normal range will

exclude the subject. Any subject excluded from the study will be provided the information concerning their exclusion and they will be referred to their primary care physician.

The subject will be scheduled to complete the four arms of the protocol at a separate time from the screening. At least one week will separate each arm of the protocol. Based on availability, protocols will be completed in the UPMC-Presbyterian Emergency Department or at the Emergency Responder Human Performance Lab (ERHPL) at 230 McKee Place, Suite 400. ERHPL is a fully equipped clinical physiology lab. Pertaining to this study, ERHPL and has the following equipment available 1) ECG monitor with non-invasive blood pressure, 2) ETCO₂, SPO₂ monitor, 3) ACLS medications and reversal agents, 4) suction, oxygen, BLS and ALS airway equipment, 5) defibrillator, and 6) a full range of temperature monitoring equipment. Both study physicians and a paramedic will be present at all procedures. In the event a patient needs to be transferred to Presbyterian Hospital, Pittsburgh EMS will be contacted through the 911 system.

Subjects will be asked to refrain from caffeine and alcohol for 24 hours prior to the protocol and from grapefruit juice for 48 hours. Grapefruit juice is known to alter cytochrome P450 metabolism while caffeine and alcohol may alter hydration status. Upon arriving for a protocol, subjects will void their bladder and be weighed.

Overview: After preparation and application of the monitoring equipment the subject will receive midazolam sedation and 30 cc/kg of either 4°C or 37°C normal saline over a 30-minute period. Depending on randomization, the subject may receive a magnesium infusion at that time. Other than the temperature of the saline and the magnesium infusion, all other procedures are identical in all four arms. Following this induction period, the subject will receive an infusion of lorazepam to maintain mild sedation. This period will allow us to determine the response of core

body temperature and the pharmacokinetics of midazolam. The protocols of interest are those using cold saline. The warm saline arms provide appropriate control groups. Following the three-hour monitoring period, the lorazepam infusion will be discontinued and a study physician will monitor the subject until it is safe for them to be discharged from the lab. The flow of the experiment is schematically shown in **Figure 2**.

Subjects will then be prepared as follows:

Intravenous Access: An 18-gauge intravenous catheter will be placed in one of the subject's hand, forearm, or antecubital veins using standard technique and universal precautions for saline infusion. A second 18- or 20-gauge intravenous catheter will be placed in the arm or hand for blood sampling.

Temperature monitoring: Core temperature will be monitored by an ingestible capsule (CorTemp) administered one hour prior to the infusion. This commercially available capsule transmits temperature to a receiving unit continuously. This capsule is FDA approved. Skin temperature will be measured by placing thermistors on the chest, back, triceps, and anterior thigh. Temperatures will be recorded every two minutes during infusion and every five minutes during recovery.

Vital signs: Heart rate, respiratory rate, ECG, ETCO₂, and SPO₂ will be measured continuously. Heart rate, respiratory rate, and SPO₂ will be documented every two minutes during infusion and every ten minutes during the follow up period. Automatic non-invasive blood pressure and the Ramsay sedation scale will be documented every five minutes during infusion and recovery.

Additional monitoring: The BIS spectral monitor (an adhesive patch placed on the subjects forehead to determine the level of sedation) and the Somanetics INVOS tissue oxygenation monitor (an adhesive patch placed over the vastus muscles to measure skeletal muscle perfusion) will be used. Surface EMG electrodes will be placed over the vastus muscles and connected to a bioamplifier to monitor for cyclical activity indicating shivering. The muscle oxygenation and EMG monitors are not related to subject safety monitoring and are used to collect preliminary data for hypothesis generation. If the logistics of one of these monitors from the implementation or data collection for the primary outcomes they will be discontinued.

Subjective measures: A 10 cm visual analog scale for discomfort will be collected every five minutes during infusion and recovery. Additionally, we will utilize a 9-point thermal sensation scale (1 = very cold, 5 = neutral, 9 = very hot) and a 4-point comfort scale (1=comfortable, 4 = very uncomfortable). Subjects will be familiarized with the scales prior to beginning the experiment.

Randomization: After screening, subjects will complete all arms of the study in a randomly generated order. Subjects will be entered sequentially into the randomization scheme as they are enrolled (**Figure 1**).

	Benzodiazepine/ Saline Infusion	Benzodiazepine + MgSO4 Saline Infusion
37°C Saline	Sedation Alone 1.	Sedation + Vasodilation 2.
4°C Saline	Sedation + Hypothermia 3.	Sedation + Vasodilation + Hypothermia 4.

Figure 1: Randomization scheme of sedation, vasodilation, and fluid temperature

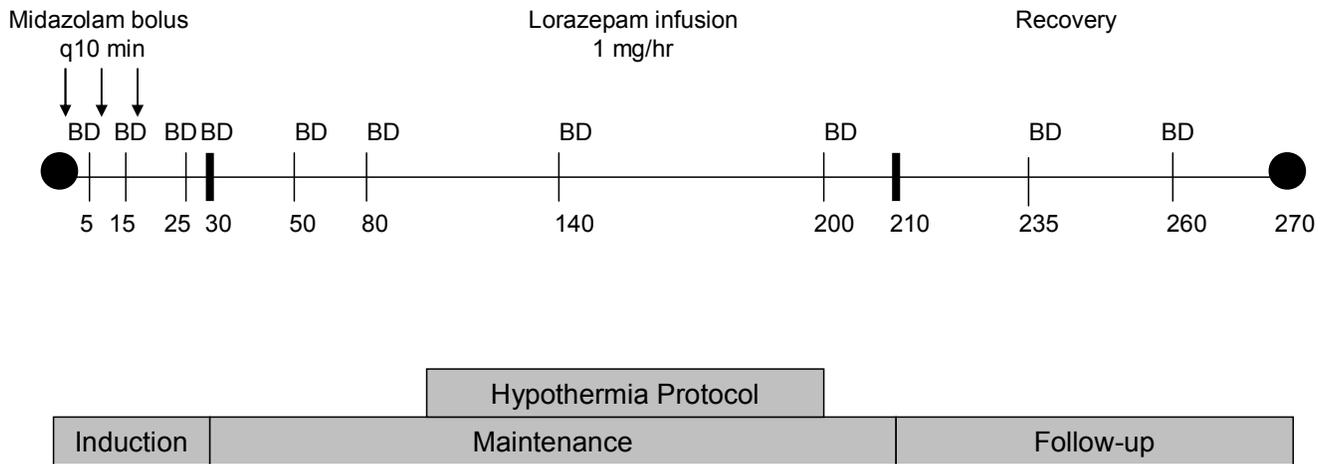
Induction period: During this 30-minute period, the subject will receive a weight based infusion of normal saline and, depending on randomization, a magnesium infusion. Subjects will receive 30mL/kg of cold (4°C) or (37°C) saline administered by peripheral IV. The total saline dose will be delivered over 30 minutes. Subjects will receive midazolam 2 mg administered intravenously just prior to initiating the infusion. Additional 2 mg doses will be administered 10 and 20 minutes into the infusion (total of 6 mg). When randomized to the appropriate arms, 4 g of magnesium sulfate will be administered during the 30-minute induction. Other studies of induced hypothermia have demonstrated that the vasodilatory effect of magnesium sulfate enhances cooling and increases subject comfort.⁹

Maintenance period: Following the 30-minute saline infusion, an infusion of lorazepam (1 mg/hr) will be initiated to maintain mild sedation for three hours. This infusion will be titrated to maintain the subject's sedation so that they respond to questions appropriately. Blood samples will be drawn and physiological monitoring will be continued during this period.

Follow up period: Following the three-hour maintenance period, the lorazepam infusion will be discontinued and the subject will be monitored for a minimum of 60 minutes. Physiological monitoring will be continued during this period and one final blood sample will be obtained.

Blood Sampling and Analysis: After administration of the third midazolam dose, blood samples will be drawn as depicted in **Figure 2**. Blood samples, (5 ml) will be obtained via a peripheral line, for a total of 40 ml of blood taken. Blood will be separated by a microcentrifuge and plasma will be stored at -80°C until further analysis.

Midazolam concentrations will be determined using liquid chromatography-mass spectrometry (LC-MS) methods as previously described.¹⁰ Briefly a 0.5-mL aliquot of plasma



BD= Blood Draw

Figure 2: Blood sampling scheme and hypothermia protocol

will be combined with 2N sodium hydroxide (0.25 mL) and internal standard (150 ng of flurazepam). Methyl *tert*-butyl ether (5 mL) will be added, and centrifuged at 3000g for 10 minutes. Samples will be evaporated to dryness under a gentle stream of nitrogen at 40°C, and reconstituted with 100 µL methanol. The samples will be transferred to a microvolume gas chromatography vials, and 4 µL will be injected into the system. The retention times for midazolam and flurazepam with use of a DB-5 (5% phenylmethylpolysiloxane) fused silica capillary column (30-m length x 0.25-mm inner diameter; 0.25-mm film thickness; J&W Scientific, Folsom, Calif) will be approximately 9.6 and 11.1 minutes, respectively. Midazolam and flurazepam (internal standard) will be monitored at mass-to-charge ratios of m/z 310 and m/z 86, respectively.

Patient safety and protocol termination: Primary endpoints for this protocol are 1) attaining a core temperature of 34°C - 35°C and maintaining the temperature for 2-3 hours or 2) if the subject requests termination. The secondary endpoint for this protocol is midazolam

pharmacokinetic alterations. If the protocol ends early for any reason the subject will continue to be monitored by a study physician until it is safe for them to be discharged.

The following criteria will result in early termination of the study: 1) If at any point in time of the study the subject is found to have a heart rate less than 40 or greater than 150, 2) systolic blood pressure less than or equal to 80mmHg or greater than 180mmHg, 3) respirations less than 7 or more than 30 per minute, 4) pulse oximetry less than 93%, 5) at any sign of arrhythmia, or 6) a sedation scale of 4. The study physician will administer flumazenil for subjects who meet these stopping criteria.

If ETCO₂ exceeds 50 mmHg during the protocol or the subject displays a Ramsay sedation score of 3, the next scheduled dose of midazolam will be replaced with a placebo saline injection and the study will continue. Additionally, subsequent doses of midazolam may be replaced with a placebo saline injection at the study physician's discretion.

In the event a subject requires airway management beyond manual jaw thrust, displays persistent hypoxia or cardiac arrhythmia, or abnormal vital signs that do not respond to flumazenil administration 911 will be called and the subject will be transferred to UPMC-Presbyterian hospital. The study physician has the option of calling 911 for other unanticipated situations requiring advanced care.

All subjects will be monitored for a minimum of 60 minutes after completing the three-hour maintenance period or until the study physician has determined they can be safely discharged. The subject must have a Ramsay Sedation Score of 2 (cooperative, oriented) before being discharged from the lab. Subjects must void their bladder and be weighed prior to discharge. No subject will be allowed to drive immediately following the protocol and will be

advised not to drive for 24 hours. Subjects must have arranged for a friend or family member to pick them up from the lab.

3.3 Data Analysis

This pilot study is descriptive in nature and will be used to determine feasibility, refine the protocol, and power a full trial. The primary statistical analysis will compare the change in temperature from baseline in the groups using repeated measures ANOVA with group and time as factors. Although unlikely to see significant changes with four subjects, significance is set at $p \leq 0.05$. Other continuous measures (e.g. heart-rate) will also be compared with ANOVA, and nonparametric tests (Kruskall-Wallis) will be used to compare ordinal or non-normally distributed data (e.g. sedation scales).

Blood samples will be maintained in the laboratory of Dr Samuel Poloyac in the University of Pittsburgh School of Pharmacy. Samples will be analyzed by Dr Poloyac, his graduate students, or assistants.

4.0 HUMAN SUBJECTS

4.1 General Characteristics

Subjects must be male aged 18 – 39 years. All races and ethnic groups are eligible for enrollment. No exclusion criteria shall be based on race, ethnicity.

4.2 Inclusion of Children

The target group for this intervention is adults with cardiovascular and cerebrovascular disease. These diseases may be qualitatively different in children. Therefore, we will not enroll subjects less than 18 years old.

4.3 Inclusion Criteria

- Subjects must be male between the ages of 18 and 39 years have no known medical problems

Exclusion Criteria

- Abnormal laboratory values or ECGs
- Those individuals who have any known history of current or previous medical problems, including but not limited to: cardiac disease, history of arrhythmia or heart murmur, hypotension or hypertension, pulmonary disease including asthma, diabetes mellitus, thyroid disease, renal disease, liver disease, neurologic disease or seizures, connective tissue disorder or Raynaud's disease, sickle cell disease, anemia, or hematologic disorders
- HIV status will not be evaluated specifically for the purpose of this study; however, those individuals who are known to be HIV positive or are otherwise immunocompromised for any reason will be excluded
- Subjects taking any medications (including herbal preparations) with the exception of allergy medication, ibuprofen or tylenol, or contraceptives. Subjects will be specifically questioned about MAO inhibitor, antidepressant, Cimetidine, and St John's Wort use.
- Individuals with a history of cardiac disease in family members below the age of forty
- Individuals with a known allergy to benzodiazepines
- Individuals with a history of long-term benzodiazepine use
- Individuals who are currently ill with a viral or other infection
- Individuals with renal insufficiency.
- Individuals who have abnormal vital signs as follows: systolic blood pressure less than 90mmHg or greater than 140mmHg, heart rate less than 40 or greater than 100, respiratory rate less than 10 or greater than 24.
- Individuals who currently smoke or use any other recreational drugs
- Individuals who are morbidly obese (BMI > 36)
- The CorTemp system is contraindicated in subjects with any history of gastrointestinal disorder, including inflammatory bowel disease, esophageal disease, hypomotility disorders, history of GI surgery, impaired gag reflex, or who weighs less than 80 pounds or is morbidly obese. Any subjects who met any of these criteria would be excluded from the study.

4.4 Recruitment Procedures

An announcement will be made in Center for Emergency Medicine, Department of Emergency Medicine, and School of Pharmacy courses and grand rounds. A recruitment flyer will be placed in common areas of the University. Volunteers will be given a phone number to

call to arrange for an appointment with an investigator. Informed consent and screening will take place at that meeting. Subjects will be fully informed of the nature of the research, including the risks and benefits of the study and their rights as a research subject prior to obtaining their signatures on the informed consent document.

No private information about the family members of the research subject will be obtained, with the exception of asking the subject if any immediate family members have any significant medical problems or if there is any history of sudden cardiac death or other cardiac disease in family members below the age of forty. No attempts will be made to contact any family members of the research subjects.

4.5 Risk/Benefit Ratio

The risk of injury or complications with this research study is moderate. The most significant risk associated with this study involves the mild hypothermia and any potential side effects of diazepam.

4.5.1 Risk associated with hypothermia:

Discomfort and shivering are the most likely side effect of the cold infusion. This risk is likely to occur in more than 25% of people (more than 25 out of 100 people). Prolonged mild induced hypothermia (temperature of 33-35°C over a period of greater than 12 hours) can affect multiple physiological systems. There may be an increase in metabolic rate and oxygen demand secondary to shivering. However, this should be easily tolerated in young healthy individuals with no cardiac problems. In a study of cardiac arrest subjects, arrhythmias were rare, even with those suffering from myocardial infarction and did not differ from control groups. Pneumonia

has occurred in those with prolonged hypothermia (greater than seven days) but is otherwise rare - expected to occur in less than 1% of people (less than 1 out of 100 people). There was no increase in pulmonary edema in cardiac arrest subjects or normal volunteers. Furthermore, these effects have all occurred in subjects who were maintained in mild hypothermia for a prolonged period of time. Several studies in normal anesthetized volunteers who received a similar infusion via central venous catheters over a period of thirty minutes showed that the hypothermia was safely achieved with none of these side effects.

4.5.2 Risks associated with midazolam administration

The most common side effects of the midazolam include drowsiness and sedation [likely - expected to occur in more than 25% of people (more than 25 out of 100 people), apnea and respiratory depression [likely - expected to occur in about 15% of people (around 15 out of 100 people)]. Subjects taking Cimetidine or with renal insufficiency may experience increased plasma levels and toxicity and are excluded from the study. Subjects may also experience involuntary movements, nausea, vomiting, hives, rash [infrequent - expected to occur in 1-10% of people (1-10 out of 100 people)]. Anti-emetic medication (Phenergan 12.5 mg IV) and anti-inflammatory medication (ibuprofen 200 mg PO) will be available for subjects experiencing nausea or headache secondary to the midazolam. Other possible and more serious effects [rare - expected to occur in less than 1% of people (less than 1 out of 100 people)] include cardiac depression or arrest, hypotension, and visual disturbances. As with any medication, an allergic reaction (e.g. rash, itching, swelling, shortness of breath) is always a risk.

Given that healthy volunteers are being studied these effects are considered unlikely.

4.5.3 Risks associated with lorazepam administration

The most common side effects of the lorazepam include sedation [likely - expected to occur in more than 25% of people (more than 25 out of 100 people)], dizziness, vertigo, weakness, or unsteadiness [infrequent - expected to occur in 1-10% of people (1-10 out of 100 people)]. Subjects taking Cimetidine or with renal insufficiency may experience increased plasma levels and toxicity and are excluded from the study. Subjects may also experience visual disturbances, sleep disturbances, depression, disorientation, headache, confusion, crying, delirium, or agitation [rare - expected to occur in less than 1% of people (less than 1 out of 100 people)]. Other possible and more serious effects [rare - expected to occur in less than 1% of people (less than 1 out of 100 people)] include either low or high blood pressure, nausea, vomiting, blurred vision, or respiratory depression. As with any medication, an allergic reaction (e.g. rash, itching, swelling, shortness of breath) is always a risk.

Given that healthy volunteers are being studied these effects are considered unlikely.

4.5.4 Risks of phenergan

This drug is being used for its FDA approved indication. The side effects from acute phenergan toxicity include deep sleep, coma, convulsions, respiratory depression, and cardiorespiratory symptoms. These side effects are rare - expected to occur in less than 1% of people (less than 1 out of 100 people). In our initial series of subjects, nausea has not been reported and phenergan has not been requested.

4.5.5 Risks of flumazenil

This drug is being used for its FDA approved indication. The side effects of flumazenil on the central nervous system include dizziness, agitation, seizures, blurred vision. Other potential side effects include nausea, vomiting, hiccups, shivering, and hypoventilation. These side effects are rare - expected to occur in less than 1% of people (less than 1 out of 100 people).

4.5.6 Risks of magnesium sulfate

The side effects of magnesium sulfate include sedation, confusion, muscle weakness, hypotension, and pulmonary edema. These side effects are rare - expected to occur in less than 1% of people (less than 1 out of 100 people) and were not reported in two previous studies utilizing magnesium sulfate to aid in hypothermia induction.^{9,11}

4.5.7 Risks of ibuprofen

This drug is being used for its FDA approved indication. The central nervous system side effects of ibuprofen include headache, dizziness, light-headedness, anxiety, and confusion. Cardiovascular side effects include hypertension, palpitations, and congestive heart failure. Other side effects include blurred vision, dry mouth, nausea, vomiting, liver damage and kidney damage. Subjects with known sensitivity or allergy to ibuprofen will not have it administered making the side effects rare - expected to occur in less than 1% of people (less than 1 out of 100 people)

4.5.8 Risk associated with medical procedures

Other potential risks include discomfort, infection, bleeding, superficial venous thrombosis, or aseptic thrombophlebitis due to the intravenous catheter insertion. This will occur rarely - expected to occur in less than 1% of people (less than 1 out of 100 people). The CorTemp temperature probe to be used carries no more risk than swallowing a pill and will pass through the subject's gastrointestinal system safely within a few days. Adverse effects from the capsule include becoming lodged in the GI tract in subjects with esophageal or intestinal disease. This will be rare - expected to occur in less than 1% of people (less than 1 out of 100 people).

There is a rare risk of redness, chafing, and irritability associated with the ECG electrodes - expected to occur in less than 1% of people (less than 1 out of 100 people).

4.5.9 Risk associated with fluid administration

For subjects with unknown heart or kidney conditions, infusion of large amounts of intravenous fluids could potentially result in congestive heart failure. This risk will be minimized by a physical examination by a physician prior to beginning the study making this a rare event. Infusion of the IV fluids could also cause abnormalities in electrolytes (substances normally found in the blood) especially in patients with kidney disease. Abnormal electrolytes can cause a variety of complications ranging from weakness, confusion, and arrhythmia. However, these complications are rare (less than 1% or less than 1 per 100) in young healthy volunteers with no previous medical problems, especially given the relatively low volume of fluids to be given.

4.5.10 Risks associated with confidentiality

The investigators will insure subject confidentiality. All subjects will be assigned a number and will be identified as such rather than by name in all subject files. Only the signed consent form will state the subject's name. All subject files will be locked in file cabinets. No record of subject information will appear in the UPMC medical records unless an adverse event occurs that would require further medical care.

4.5.11 Benefits

There will be no direct benefit to the subject for participation in this research study.

4.5.12 Data Safety Monitoring Plan

The investigators will meet weekly and after each patient enrollment to ensure the data integrity and subject confidentiality has been maintained. Adverse events, a need to modify the protocol, and changes in the risk profile will be reported in compliance with Chapter 3 of the IRB manual. In addition to subject accrual, the following data will be accounted for at these meetings: temperature measures, medication administration, adverse events, vital signs, IV administration start and stop times. Data and Safety Monitoring reports will be submitted to the IRB (at time of annual renewal) and the investigators will comply with the IRB policy for reporting adverse events as outlined in Chapter 3.0, sections 3.4 and 3.5 of the IRB Reference Manual.

5.0 COST AND PAYMENTS

5.0.1 Costs

None of the services and/or procedures (*labs, medications, temperature monitoring*) provided for this research study will be billed to the subject or to the subject's health insurance.

5.0.2 Payments

Subjects will be paid \$400 for completing the entire study. A partial payment of \$100 per protocol completed will be provided for subjects who withdraw before completing all protocols. Subjects who are not cleared for participation by the study physician or who withdraw prior to beginning the infusion will not be paid.

APPENDICES

6.1 Qualifications of Investigators:

Clifton Callaway, MD, PhD, is an Assistant Professor for the University of Pittsburgh Department of Emergency Medicine and attending physician in Emergency Medicine at Presbyterian Hospital.

David Hostler, PhD, NREMT-P, is the Director of Research for the University of Pittsburgh Affiliated Residency in Emergency Medicine. His doctorate was obtained in the field of exercise physiology and has extensive experience in conducting human physiology experiments.

Samuel Poloyac, PhD, Pharm D is an Assistant Professor of Pharmaceutical Sciences at the University of Pittsburgh.

Michael Tortorici, PharmD is a doctoral candidate in clinical pharmacy graduate program at the University of Pittsburgh.

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B.2 CONSENT FORM

CONSENT TO ACT AS A SUBJECT IN AN EXPERIMENTAL STUDY

TITLE: Therapeutic hypothermia induction and pharmacokinetics in healthy human subjects

INVESTIGATORS: David Hostler, PhD, NREMT-P, CSCS

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Source of Support: Department of Emergency Medicine

Why is this research being done?

You are being asked to participate in a research study in which we will study the effects in healthy people of mild induced hypothermia (i.e., lowering of body temperature) using intravenous (IV) fluids and mild sedation. Hypothermia has been shown to protect the brain in a variety of emergency medical situations, including when the heart stops suddenly (cardiac arrest) and strokes. IV fluids are an intervention that can easily be started by paramedics early in the treatment of these patients. However, cooling the body causes shivering which may create heat making it difficult to make a patient cool enough to see a benefit. We are therefore determining whether hypothermia can be quickly and effectively induced and maintained in normal subjects after administering cold IV fluid when using midazolam (Versed) and lorazepam (Ativan) to reduce the shivering that occurs when the body becomes cold. We also want to investigate if hypothermia (lowering of body temperature) affects the ability of the liver to breakdown the drugs you are going to be given. This is important because it will help us to understand how the liver works when patients' body temperature is cooled. This information will also help us to figure out the right dose of medications to give to patients treated with hypothermia.

In this study, we will infuse cold 39°F (4°C) or warm 98.6°F (37°C) IV fluids into a vein into your arm on four separate occasions while we measure your core body temperature (the temperature in the center of your body). You will either be given midazolam (Versed) at three specific times during the study and a continuous infusion of lorazepam (Ativan). Midazolam (Versed) and lorazepam (Ativan) is approved by the Food and Drug Administration (FDA) as a sedative. On two visits, we will also give you the medication magnesium sulfate. This medicine will cause your arteries and veins to open slightly bringing more blood to the skin. Magnesium

sulfate is known to relax the muscle cells in your arteries and veins but has not been approved by the FDA for this use.

We will monitor your other vitals signs including heart rate, respiration (breathing) rate, blood pressure, end-tidal CO₂ (the amount of carbon dioxide you exhale) and pulse oximetry (an indicator of how much oxygen your blood is carrying). We will also take eight (8) teaspoonful of blood during the study for a total of 40 ml teaspoonful of blood. Finally, we will also assess the amount of discomfort caused by the fluids.

Who is being asked to take part in this research study?

People invited to participate in this study must be males between the ages of 18 and 39 years of age. The study requires five visits to complete. One visit will be required for the screening and the physician exam. Four additional visits are required to complete the four experiments.

If you meet any of the following conditions you will also be excluded from the study:

- If you have any known history of current or previous medical problems, including but not limited to: heart disease, history of abnormal heart rhythm (arrhythmia) or heart murmur, low blood pressure, high blood pressure, lung disease including asthma, diabetes mellitus, thyroid disease, kidney disease, liver disease, nerve disease or seizures, connective tissue disorder or Raynaud's disease, sickle cell disease, anemia, or other blood disorders
- If you are known to have the human immunodeficiency virus (HIV positive) or are otherwise have difficulty fighting infection (weakened immune system).
- If you take any medications (including herbal preparations) with the exception of allergy medication, ibuprofen or tylenol.
- If you take any of the following medications or over-the-counter items: MAO inhibitors (such as Parnate or Nardil) or any other antidepressant, St John's Wort, or Cimetidine (Tagamet). Participating in the study while on these medicines could cause serious illness or death.
- If there is a history of heart disease in family members below the age of forty
- If you have a known allergy to midazolam (Versed) or lorazepam (Ativan), or a history of other narcotic allergy
- If you have a history of long term benzodiazepine use
- If you are currently ill
- If you have kidney problems or renal insufficiency

- If you have abnormal vital signs including systolic blood pressure (the first number given when your blood pressure is taken) less than 90mmHg or greater than 140mmHg, heart rate less than 40 or greater than 100, or a respiratory rate (the number of times you breathe in one minute) less than 10 or greater than 24.
- If you currently smoke or use any other recreational drugs
- If you are morbidly obese (very overweight – Body Mass Index > 36)
- An abnormal amount of sodium (salt) measured in your blood
- If you have any history of stomach (gastrointestinal) disorder, including inflammatory bowel disease, esophageal disease, hypomotility (slow digestion) disorders, history of GI surgery, impaired gag reflex
- You weigh less than 80 pounds

This study is being performed on a total of 4 subjects.

What procedures will be performed for research purposes?

If you decide to take part in this research study, you will undergo the following procedures that are not part of standard medical care:

Screening Procedures:

Procedures to determine if you are eligible to take part in a research study are called “screening procedures”. For this research study, the screening procedures include:

1. A brief medical evaluation. This will include questions about previous and current medical problems, medications and allergies, and family history. A short physical exam will also be performed, to assess for the presence of any cardiac or respiratory abnormalities, or any other problems.
2. We will assess your body fat by using calipers to measure the skin in three places (men – chest, abdomen, and thigh; women – arm, hip, and thigh).
3. An EKG (electrocardiogram) of your heart will be performed to assess for any underlying cardiac disorders. This involves placing twelve stickers on your chest, arm and legs and recording your heart’s electrical activity for about twenty seconds.
4. A blood sample will be collected from a vein in your arm to test for thyroid, liver, and blood diseases. The following tests will be performed on this blood: sodium and potassium level, thyroid serum hormone, SGOT, SGPT, alkaline phosphatase, billirubin (liver proteins),

BUN, creatinine, and a complete blood count. A maximum of 30cc (2 tablespoon) of blood will be collected.

These procedures will take about sixty minutes total and will be performed in the Department of Emergency medicine offices. Once you have completed the screening and been approved by the study physician you will be scheduled for the experimental protocol.

Experimental Procedures:

If you qualify to take part in this research study, you will undergo the experimental procedures listed below four times, each time separated by at least one week. Each time you arrive you will have medical monitors attached to follow your temperature and vital signs. You will receive the medication midazolam (Versed) three times. This medicine might make you sleepy. You will also receive IV fluids for 30 minutes while you get this medication. On two visits the fluid will be cold and on two visits it will be warm. On two of the four visits you will also receive the medication magnesium sulfate. All the medications will be given through a vein in your arm.

After 30 minutes you will receive a second medication (lorazepam or Ativan) that may continue the sleepy feeling. During this time we will draw blood samples and monitor your temperature and vital signs for three hours. Following this the medications will be stopped and you will be watched by a physician until you wake up and have recovered.

These procedures will take place in either the Presbyterian Hospital emergency department or the Emergency Responder Human Performance Lab at the Department of Emergency Medicine. The experimental procedures will take approximately 5-6 hours including preparation, the experiment, and follow up. Before each protocol, you should not consume

alcohol or caffeine for 24 hours or grapefruit juice for 48 hours as these may interfere with the study.

To ensure all subjects are cooled equally during the study, males will be asked to wear short pants and no shirt during the study

All subjects will have the following procedures performed

1. We will place several stickers onto your chest, arms, and legs which will enable you to be connected to a cardiac monitor and a temperature monitor. This will provide a constant assessment of your heart rate and rhythm throughout the test and skin temperature. A blood pressure cuff will be placed on your arm and another monitor will be placed upon your index finger to assess your pulse oximetry (an indication of how much oxygen is being carried by your blood). A small plastic tube (cannula) will be placed at the entrance of your nose to measure your exhaled breath.

2. You will be given a capsule approximately the size of a large Tylenol to swallow which contains a temperature sensor and transmitter. It will detect your core temperature (i.e., in your stomach and gastrointestinal tract) and transmit this information to a sensor outside of your body. This will pass through your GI tract and be eliminated several days later when you move your bowels. This capsule is FDA approved.

3. You will be weighed after you empty your bladder.

4. An 18-gauge intravenous catheter (IV) will be placed into one of your antecubital veins (in the crease of your arm formed by your elbow) or in a forearm or hand vein. The amount of fluid will be adjusted for your body weight (30mL/kg or 0.5 oz per pound), about 2 liters (about 0.5 gallons) for an average sized adult. One teaspoonful of blood will be taken from this catheter in your vein for a total of ten (10) times throughout the study for a total of fifty ml. These samples will be stored in Dr. Poloyac's lab in the School of Pharmacy and will be

analyzed by Dr. Poloyac, his graduate students, or assistants. The samples will be labeled with a number. The list linking the number to you and your data will be kept in a separate location. These samples will not be shared with other researchers.

5. Stickers will be placed on your forehead and thighs to measure and connected to monitors to determine how drowsy you become and how much oxygen is in your muscles.

6. You will receive three doses of midazolam (Versed) ten minutes apart. IV fluids will be infused through the IV at a rate of 30cc/kg for thirty minutes. During this time, we will monitor your temperature via the capsule you swallowed every two minutes. We will also record your heart rate every two minutes from the monitors. Your blood pressure will be checked every five minutes in the arm opposite of the IV.

7. You will also be asked to assess your level of discomfort using a number of simple scales. This means that you will be shown a series of numbers with explanation of temperature and discomfort. These assessments will be performed immediately prior to starting the fluids, every five minutes during the test, and every ten minutes for another half an hour following the test.

8. Fluids will stop approximately 30 minutes after beginning. At that time, you will be given an infusion of lorazepam (Ativan) to maintain your relaxed state for 3 hours. During this time we will monitor your temperature and vital signs. We will draw a tablespoon of blood seven times during this period. Following this period, the medications will be stopped and you will be monitored for at least one hour while you recover.

The study will be ended early and you will be given medication to reverse the sedation if any of the following occur:

- Your core temperature goes below 93.2°F (34°C)
- At your request
- If your vital signs become significantly abnormal, including a heart rate less than 40 beats per minute (bpm) or greater than 150 bpm, systolic blood pressure equal or less than 80 or greater than 180, or respirations less than 7 per minute or more than 30 per minute.

- If you become confused or sleepy to the point that you cannot be easily awoken
- The pulse oximeter reading becomes less than 93% (there is a decrease in the level of oxygen carried by the blood).
- Any sign of cardiac arrhythmia (irregular or abnormal heart rhythm)

Monitoring/Follow-up Procedures:

Procedures performed to evaluate the effectiveness and safety of the experimental procedures are called “monitoring” or “follow-up” procedures. For this research study, the monitoring/follow-up procedures include:

1. For a minimum of 60 minutes after stopping the IV fluids, we will monitor your temperature, heart rate, blood pressure, and pulse oximetry, as well as your level of discomfort using the scale described above.
2. You will be given warm blankets and or liquids as desired.
3. You will be weighed after you empty your bladder.
4. If after 60 minutes, your temperature is above 96.8°F (36°C), your vital signs are all within normal limits, you feel physically comfortable, and the study physician has determined you are well enough to leave you may leave with no further follow-up required.
5. You will not be permitted to drive immediately following the study and should not drive for 12 hours after completing the study. You must have alternate transportation available before you begin the study.

What are the possible risks, side effects, and discomforts of this research study?

As with any experimental procedure, there may be adverse events or side effects that are currently unknown and certain of these unknown risks could be permanent, severe, or life threatening.

The possible risks of this research study may be due to the decrease in core body temperature (hypothermia), the medications given, and the placement of the IV line and blood tests.

Risks of Mild Induced Hypothermia:

The most common effects of hypothermia include discomfort or pain (especially in fingers and toes) and shivering. Chances are likely (more than 25%, or 25 in 100) that you will experience shivering and/or discomfort while receiving the saline.

Long periods of mild cooling (temperature of 91.4-95°F (33-35°C) over a period of greater than 12 hours) can affect different parts of the body. There may be an increase in metabolism (the rate at which your body burns fuel and oxygen) required during shivering. Pneumonia (a lung disease) has occurred in those with prolonged hypothermia (greater than seven days) but is otherwise rare - expected to occur in less than 1% of people (less than 1 out of 100 people). The period of cooling in this study is less than one hour making this rare - expected to occur in less than 1% of people (less than 1 out of 100 people).

Risks associated with midazolam administration:

The most common side effects of the midazolam include drowsiness, sedation [likely - expected to occur in more than 25% of people (more than 25 out of 100 people)], apnea (brief pauses in your breathing) or a decrease in the rate of your breathing, [likely - expected to occur in about 15% of people (around 15 out of 100 people)], involuntary movements, nausea, vomiting, hives, rash, [infrequent - expected to occur in 1-10% of people (1-10 out of 100 people)]. You may also experience visual disturbances [rare - expected to occur in less than 1% of people (less than 1 out of 100 people)]. Other possible and more serious effects [rare - expected to occur in less than 1%

of people (less than 1 out of 100 people)] include cardiac depression (poor heart function) or arrest, hypotension (low blood pressure). As with any medication, an allergic reaction (e.g. rash, itching, swelling, shortness of breath) is always a risk.

A physician and emergency drugs and equipment will be readily available should you experience any adverse reactions from administration of the study drug. You will be offered the medication Phenergan if you become nauseated during the study or ibuprofen if you have a headache. If you become too drowsy you will be given the medication flumazenil.

The medicines that may be provided to treat over sedation, nausea, and headache carry their own risks.

Risks of lorazepam (Ativan) administration:

The most common side effects of the lorazepam include sedation [likely - expected to occur in more than 25% of people (more than 25 out of 100 people)], dizziness, vertigo, weakness, or unsteadiness [infrequent - expected to occur in 1-10% of people (1-10 out of 100 people)]. You may also experience visual disturbances, sleep disturbances, depression, disorientation, headache, confusion, crying, delirium, or agitation [rare - expected to occur in less than 1% of people (less than 1 out of 100 people)]. Other possible and more serious effects [rare - expected to occur in less than 1% of people (less than 1 out of 100 people)] include either low or high blood pressure, nausea, vomiting, blurred vision, or respiratory depression. As with any medication, an allergic reaction (e.g. rash, itching, swelling, shortness of breath) is always a risk.

A physician and emergency drugs and equipment will be readily available should you experience any adverse reactions from administration of the study drug. You will be offered the medication Phenergan if you become nauseated during the study or ibuprofen if you have a headache. If you become too drowsy you will be given the medication flumazenil.

The medicines that may be provided to treat over sedation, nausea, and headache carry their own risks.

Risks of phenergan (promethazine) administration:

This drug is being used for its FDA approved indication. Phenergan will be administered if you become nauseated during the protocol. This is the normal use for this medication. The side effects from phenergan include deep sleep, coma, convulsions, slow breathing, abnormal heartbeat, high blood pressure, or low blood pressure. These side effects are rare - expected to occur in less than 1% of people (less than 1 out of 100 people).

Risks of flumazenil (Romazicon):

This drug is being used for its FDA approved indication. Flumazenil will be given if you become too sedated. This is the normal use for this medicine. The side effects of flumazenil include dizziness, agitation, seizures, blurred vision. Other potential side effects include nausea, vomiting, hiccups, shivering, and slow breathing. These side effects are rare - expected to occur in less than 1% of people (less than 1 out of 100 people).

Risks of ibuprofen (Advil):

This drug is being used for its FDA approved indication. Ibuprofen will be given if you develop a headache. This is the normal use of this medicine. The side effects of ibuprofen include headache, dizziness, light-headedness, anxiety, and confusion. Side effects of the heart include high blood pressure, palpitations, and heart failure. Other side effects include blurred vision, dry mouth, nausea, vomiting, liver damage and kidney damage. These side effects rare - expected to occur in less than 1% of people (less than 1 out of 100 people)

Risks of the Intravenous Catheter Placement:

Bruising and soreness occur infrequently and infection, bleeding, or a condition known as aseptic thrombophlebitis (blood clots in the vein) may rarely occur as a result of the needle sticks to obtain blood from your vein or to start an IV to give fluids and medication. These risks are rare (less than 1% or less than 1 per 100) and they are minimized by having blood drawn by trained physicians and paramedics. There is a risk you could pass out while having your blood drawn [infrequent - expected to occur in 1-10% of people (1-10 out of 100 people)]. You will sit in a special chair to ensure you do not fall if this happens. You will be placed laying down, face up and have your feet elevated until you recover.

Risks of the IV Fluids:

If you have an unknown heart or kidney condition, infusion of large amounts of intravenous fluids could potentially result in congestive heart failure, or a build up of fluid in your lungs and other tissues. This could result in difficulty breathing and potentially further damage to your heart and kidneys. You will be given a physical examination by a physician prior to beginning the study making this a rare event. Infusion of the IV fluids could also cause abnormalities in electrolytes (substances normally found in the blood) especially in patients with kidney disease. Abnormal electrolytes can cause a variety of complications ranging from weakness, confusion, and arrhythmia. However, these complications are rare (less than 1% or less than 1 per 100) in young healthy volunteers with no previous medical problems, especially given the relatively low volume of fluids to be given.

Other potential risks:

The CorTemp temperature probe to be used carries no more risk than swallowing a pill and will pass through your stomach and intestines safely within a few days. There is a risk the capsule

will become lodged in your digestive system if you have a disease of the esophagus or intestine. This is rare and expected to occur in less than 1% of people (less than 1 out of 100 people). There is a rare risk of redness, chafing, and irritability associated with the ECG electrodes - expected to occur in less than 1% of people (less than 1 out of 100 people).

What are possible benefits from taking part in this study?

You will likely receive no direct benefit from taking part in this research study.

If I agree to take part in this research study, will I be told of any new risks that may be found during the course of the study?

You will be promptly notified if, during the conduct of this research study, any new information develops which may cause you to change your mind about continuing to participate. Furthermore, you will be informed of any findings or health concerns that may be found during your screening history and physical, screening blood work, or during cardiac monitoring.

Will my insurance provider or I be charged for the costs of any procedures performed as part of this research study?

The study will pay for research-only costs. Neither you, nor your insurance provider, will be charged for the costs of any of the procedures performed for the purpose of this research study (i.e., the Screening Procedures, Experimental Procedures, or Monitoring/Follow-up Procedures described above).

Will I be paid if I take part in this research study?

You will be paid a total of \$400 if you pass the screening and complete all four scheduled sessions. You will be paid \$100 for each session you complete if you do not complete all four sessions. If you are found to be ineligible during the initial exam, pregnancy test, or EKG screen, you will not be paid.

Who will pay if I am injured as a result of taking part in this study?

University of Pittsburgh researchers and their associates who provide services at the UPMC recognize the importance of your voluntary participation in their research studies. These individuals and their staffs will make reasonable efforts to minimize, control, and treat any injuries that may arise as a result of this research. If you believe that you are injured as a result of the research procedures being performed, please contact immediately the Principal Investigator or one of the co-investigators listed on the first page of this form.

Emergency medical treatment for injuries solely and directly related to your participation in this research study will be provided to you by the hospitals of the UPMC. It is possible that the UPMC may bill your insurance provider for the costs of this emergency treatment, but none of these costs will be charged directly to you. If your research-related injury requires medical care beyond this emergency treatment, you will be responsible for the costs of this follow-up care unless otherwise specifically stated below. You will not receive any monetary payment for, or associated with, any injury that you suffer in relation to this research.

Who will know about my participation in this research study?

Any information about you obtained from this research will be kept as confidential (private) as possible. All records related to your involvement in this research study will be stored in a locked

file cabinet. Your identity on these records will be indicated by a case number rather than by your name, and the information linking these case numbers with your identity will be kept separate from the research records. You will not be identified by name in any publication of the research results unless you sign a separate consent form giving your permission (release).

Will this research study involve the use or disclosure of my identifiable medical information?

This research study will not access your medical records nor will information from this research study be placed in your medical records. However, a medical record could be generated in the unlikely event a complication occurs.

Who will have access to identifiable information related to my participation in this research study?

In addition to the investigators listed on the first page of this authorization (consent) form and their research staff, the following individuals will or may have access to identifiable information related to your participation in this research study:

Authorized representatives of the University of Pittsburgh Research Conduct and Compliance Office may review your identifiable research information for the purpose of monitoring the appropriate conduct of this research study.

In unusual cases, the investigators may be required to release identifiable information related to your participation in this research study in response to an order from a court of law. If the investigators learn that you or someone with whom you are involved is in serious danger or

potential harm, they will need to inform, as required by Pennsylvania law, the appropriate agencies.

Authorized representatives of the sponsor of this research study, the University of Pittsburgh General Clinical Research Center, may review and/or obtain identifiable information related to your participation in this research study for the purpose of monitoring the accuracy and completeness of the research data and for performing required scientific analyses of the research data. Authorized representatives of the study sponsor may also be present during your participation in the procedures performed as part of this research study. While the study sponsor understands the importance of maintaining the confidentiality of your identifiable research and medical information, the UPMC and University of Pittsburgh cannot guarantee the confidentiality of this information after it has been obtained by the study sponsor. The investigators involved in the conduct of this research study may receive funding from the sponsor to perform the research procedures and to provide the sponsor with identifiable research and medical information related to your participation in the study.

Authorized representatives of the UPMC hospitals or other affiliated health care providers may have access to identifiable information related to your participation in this research study for the purpose of (1) fulfilling orders, made by the investigators, for hospital and health care services (e.g., laboratory tests, diagnostic procedures) associated with research study participation; (2) addressing correct payment for tests and procedures ordered by the investigators; and/or (3) for internal hospital operations (i.e. quality assurance).

For how long will the investigators be permitted to use and disclose identifiable information related to my participation in this research study?

The investigators may continue to use and disclose, for the purposes described above, identifiable information related to your participation in this research study for a minimum of 5 years and for as long (indefinite) as it may take to complete this research study.

May I have access to my medical information that results from my participation in this research study?

No medical information will be placed into your medical records.

Is my participation in this research study voluntary?

Your participation in this research study, to include the use and disclosure of your identifiable information for the purposes described above, is completely voluntary. (Note, however, that if you do not provide your consent for the use and disclosure of your identifiable information for the purposes described above, you will not be allowed, in general, to participate in the research study.) Whether or not you provide your consent for participation in this research study will have no effect on your current or future relationship with the University of Pittsburgh. Whether or not you provide your consent for participation in this research study will have no effect on your current or future medical care at a UPMC hospital or affiliated health care provider or your current or future relationship with a health care insurance provider.

These doctors are involved as investigators in this research study. As both doctors and research investigators, they are interested both in your medical care and the conduct of this research study. Before agreeing to participate in this research study, or at any time during your study participation, you may discuss your care with another doctor who is not associated with this research study. You are not under any obligation to participate in any research study.

May I withdraw, at a future date, my consent for participation in this research study?

You may withdraw, at any time, your consent for participation in this research study. Any identifiable research or medical information recorded for, or resulting from, your participation in this research study prior to the date that you formally withdrew your consent may continue to be used and disclosed by the investigators for the purposes described above.

To formally withdraw your consent for participation in this research study you should provide a written and dated notice of this decision to the principal investigator of this research study at the address listed on the first page of this form.

Your decision to withdraw your consent for participation in this research study will have no effect on your current or future relationship with the University of Pittsburgh. Your decision to withdraw your consent for participation in this research study will have no effect on your current or future medical care at a UPMC hospital or affiliated health care provider or your current or future relationship with a health care insurance provider.

If I agree to take part in this research study, can I be removed from the study without my consent?

It is possible that you may be removed from the research study by the researchers if, for example, your pregnancy test proves to be positive. If you are withdrawn from participation in this research study due to a positive pregnancy test, or any concerns on your physician exam, you will not undergo the subsequent study protocol.

VOLUNTARY CONSENT

All of the above has been explained to me and all of my current questions have been answered. I understand that I am encouraged to ask questions about any aspect of this research study during the course of this study, and that such future questions will be answered by the researchers listed on the first page of this form.

Any questions which I have about my rights as a research participant will be answered by the Human Subject Protection Advocate of the IRB Office, University of Pittsburgh (1-866-212-2668).

By signing this form, I agree to participate in this research study. A copy of this consent form will be given to me.

Participant's Signature

Date

CERTIFICATION of INFORMED CONSENT

I certify that I have explained the nature and purpose of this research study to the above-named individual(s), and I have discussed the potential benefits and possible risks of study participation. Any questions the individual(s) have about this study have been answered, and we will always be available to address future questions as they arise.”

Printed Name of Person Obtaining Consent

Role in Research Study

Signature of Person Obtaining Consent

Date

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