

**Evaluation of the effects of therapeutic hypothermia and cardiac  
arrest on specific cytochrome P450 isoform activity**

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## **PREFACE**

The purpose for any drug metabolism and pharmacokinetic (DMPK) study is to evaluate the characteristics of absorption, distribution, elimination and excretion from the body for a specific drug under certain conditions. The knowledge of DMPK is essential in understanding and evaluating drug concentrations and responses to ensure effective and safe drug use.

I am particularly interested in evaluating the effects of the changes in body temperature (30-35°C, mild therapeutic hypothermia) on drug metabolism and hepatic metabolizing enzyme activities. Our body temperature has a fundamental influence on nearly all aspects of physiological function. Forced cooling by therapeutic hypothermia potentially has side effects on multiple organ functions, including the activity of metabolic enzymes and therefore, has potential effects on the metabolism and elimination of xenobiotic substances. The work presented in this dissertation has been focused on a preclinical and clinical studies aiming to determine the activities of multiple drug metabolizing enzymes under therapeutic hypothermia conditions after critical illness. This work also focuses on the prediction of drug concentration under altered body temperature. Our findings are relevant to clinical and preclinical pharmacokinetic analysis study, resuscitation pharmacology, and clinical pharmacology. Furthermore, results from this study are expected to provide insightful information for the clinicians in their estimation of the effect of temperature alterations on drug metabolism and disposition. Furthermore, results from this study are expected to provide insightful information for the clinicians in their estimation of the effect of temperature alterations on drug metabolism and disposition.

This work can be considered as the expansion of the current knowledge and advancement in topics of evaluating the effect of therapeutic hypothermia as well as cardiac arrest on drug metabolism. Both clinical and preclinical studies were carried out. This study has contributed to our understanding of the effect of hypothermia and cardiac arrest on the activity of specific CYP isoform, advanced cocktail bio-analytical assays development, and the utilization of microdosing PK methodology for critical ill patients. We have also performed population pharmacokinetic modeling and simulation analysis, in which the body temperature was considered as a possible statistical covariate of drug metabolism. Strong correlations have been found by establishing the mathematic PK model to best fit the observed data and proposed the physiological relationships. Results of this study are expected to provide rationale for drug dose adjustment to ensure a safer and more effective pharmacotherapy in this highly vulnerable patient population. We have elucidated the effects of hypothermia based on specific drug metabolic pathway, patients' disease state, and specific temperature treatment and duration.

Throughout my dissertation work, I have grown tremendously with the help from my dear family, friends, and my brilliant colleagues and mentors. I received a lot of help from many people in the field and therefore, I am very grateful, appreciative and thankful, especially to: my advisor Dr. Samuel Poloyac for his guidance and support throughout my tenure in his laboratory, to all my committee members Dr. Patrick Kochanek, Dr. Clifton Callaway, Dr. Robert Bies, and Dr. Wen Xie, from whom I have benefited greatly from their experience and their knowledge. I'd like to acknowledge and thank the research staffs from the School of Pharmacy, Emergency Medicine and Safar Center whom have all helped me thru the years, and I would like to share this achievement with my husband John, my parents and my sister and her family. Looking back now, I realize just how much I truly enjoyed this learning experience and I want to encourage

more students and scientists to further investigate disease related and treatment related drug metabolism and disposition.

# **Evaluation of the effects of therapeutic hypothermia and cardiac arrest on specific cytochrome P450 isoform activity**

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University of Pittsburgh, 2011

## **ABSTRACT:**

Therapeutic hypothermia (TH, 32-35°C) mediated neuroprotection after brain ischemia has been demonstrated by both preclinical and clinical studies. Studies to identify the effects of mild hypothermia on CYP450 metabolism in humans are limited and the translational significance of the observations in the rat model remains to be identified. The goal of this study is to evaluate the effects of therapeutic hypothermia and relevant disease model cardiac arrest (CA) on hepatic drug metabolism. Specifically, this study evaluated the effects of therapeutic hypothermia on specific CYP450-mediated drug metabolism in preclinical and in translational clinical studies.

There are several conclusions can be made based on our study results. Mild hypothermia and cardiac arrest alter CYP activity in an isoform specific manner. Magnitude of the reduction is likely temperature, and extraction ratio specific. In animal model, the combination of hypothermia (33°C) and CA was most likely to be associated with isoform specific decrease of enzyme activities with greater changes observed for CYP3A and CYP2E1. Hypothermia decreased the volume of distribution of multiple probe substrates. In healthy volunteers, we found significant correlation between temperature and the clearance of CYP3A probe drug midazolam. Short duration hypothermia studies with hepatically eliminated drugs suggest ~11% reduction in clearance per °C change. Microdosed cocktail probes are likely to be very useful in

PK study design in critically ill patients due to the potential linearity of PK of probe drugs and no drug-drug interaction in the cocktail combination. In addition, our studies show that therapeutic hypothermia inhibited phenytoin metabolism in neonates with hypoxic ischemic encephalopathy,  $K_m$  increase ~19% per °C and this likely due to the reduced activity of CYP2C9/CYP2C19, and reduced affinity between substrates and enzymes. In addition, effect of cooling on receptor dynamic response is unknown on the current data.

In conclusion, our results have shown the effect of CA and hypothermia with interaction on isoform specific activity. Given the prominent role of mild hypothermia in the management of patients with CA, further translational studies using clinically relevant drugs and pharmacokinetics-pharmacodynamics modeling are vital for validation and prediction of drug dosing in different disease and temperature states.

**Key words:** Therapeutic hypothermia, cardiac arrest, drug metabolism, critical care medicine, pharmacokinetics, in vivo probes, cytochrome P450, UPLC/MS/MS, population pharmacokinetic modeling

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Hearts and brains, too good to die

— Dr. Peter Safar, Father of CPR

There's a way to do it better - find it.

— Thomas A. Edison

## 1.0 INTRODUCTION

### 1.1 CARDIAC ARREST AND BRAIN ISCHEMIA

#### **1.1.1 Cardiac arrest and global ischemic injury**

Cardiac arrest (CA) is the cessation of cardiac mechanical activity as confirmed by the absence of signs of circulation. More than 80% of sudden cardiac deaths occur in people with coronary artery disease. It is the number one natural killer among adults in the United States. Approximately half of all cardiac deaths can be classified as sudden cardiac arrest which accounting for approximately 300,000-450,000 deaths per year. Of these, 20-38% patients have ventricular fibrillation or ventricular tachycardia as the first recorded rhythm (Nichol 1999; Vaillancourt 2000). With the help of advanced cardiopulmonary resuscitation (CPR), only 37% of in-hospital cardiac arrests and 9% of out-of-hospital cardiac arrests can survive (Wright et al., 2006).

Brain injury starts to occur just about 4-6 minutes after the cardiac arrest. Brain injury can occur as a consequence of a reduction in cerebral blood flow (CBF) and subsequently loss of oxygen supply. The interruption of the blood flow and oxygen supply in the brain results in nutrition supply shortage, tissue death and loss of brain function, and severe neurological damage ((Dearden 1985; Hertz 1981; Arai et al., 1984; Guercio et al., 1965). Brain ischemia in critical care medicine is associated with high mortality. Of all CA survivors, only between 3 and 10% are able to resume their former lifestyles and half experience persistent brain dysfunction (Wright et al., 2006).

### **1.1.2 Pathophysiology of brain ischemic injury**

After the interruption of circulation, multiple mechanisms of ischemic injury occur. Neuronal ischemic injury occurs rapidly with significant structure changes being observed within minutes. Siesjo et al. has reported that within 20 seconds of interruption of blood flow to the mammalian brain, under the conditions of normothermia, the EEG activity disappears, probably as a result of the failure of high energy metabolism. Within 5 minutes, high energy phosphate levels have virtually disappeared (ATP depletion) and profound disturbances in cell electrolyte balance start to occur (Siesjo et al., 1981).

The loss of cellular high energy compounds during ischemia causes the loss of the  $\text{Na}^+ - \text{K}^+$  gradient, thus disrupting the cellular calcium homeostasis. This causes a massive and rapid influx of calcium into the cell. During ischemia, the NMDA (N-methyl-d-aspartate) receptor has been activated and they will also facilitate calcium entering into neurons (Berdichevsky 1983). Elevated intracellular calcium activates membrane phospholipases and protein kinases, leading to the production of free fatty acids including arachidonic acid. The degradation of membrane by phospholipase further reduces the efficacy of calcium pump, thereby leading to further calcium overload (Wolfe 1982).

In addition, cerebral recovery from more than 5 minutes of cardiac arrest is greatly hampered by the secondary derangements of multiple organ systems after reperfusion (Safar 1985). After re-introduction of the oxygen, the free fatty acids are metabolized into reactive oxygen species. Also, during ischemia, the hydrolysis of ATP via AMP leads to an accumulation of hypoxanthine. Increased intracellular calcium level enhances the conversion of xanthine

dehydrogenase to xanthine oxidase. Reactive oxygen species (ROS) will be generated by xanthine oxidase during reperfusion and oxygenation. Therefore, increased levels of several free radical species cause cell necrosis, apoptosis, DNA damage, altered mRNA selection and capillary membranes damage (Darwin, 1995; Tien et al., 1982; McCord 1985; Kleihues et al., 1974). Calcium loading and free radical generation are the major contributors to mitochondrial structure changes (Frenkel et al., 1980). Mitochondrial stress leads to the release of cytochrome C, further inducing apoptosis and neuronal death.

With the understanding of the mechanisms of the pathophysiology of cerebral ischemia, many possible pharmacological or non-pharmacological interventions have been tested to reduce brain damage. These interventions include: 1) calcium channel blockers, 2) reducing free radical, by pre- or post-insult treatment with nutritional antioxidant such as Vitamin E, selenium, vitamin C and beta carotene, 3) phospholipase inhibitor, 4) protection against the deleterious effects of excitotoxicity, NMDA glutamate receptor inhibitor, 5) increasing perfusion pressure, and 6) inhibition of inflammation (Darwin 1995).

These possible interventions have been tested in the past decades but so far all of them have failed to demonstrate the neurological benefit after cardiac arrest in clinical trials. It has been proposed that the failure of individual agents is due to the fact that they typically target a single pathway in a multifactorial process of brain injury.

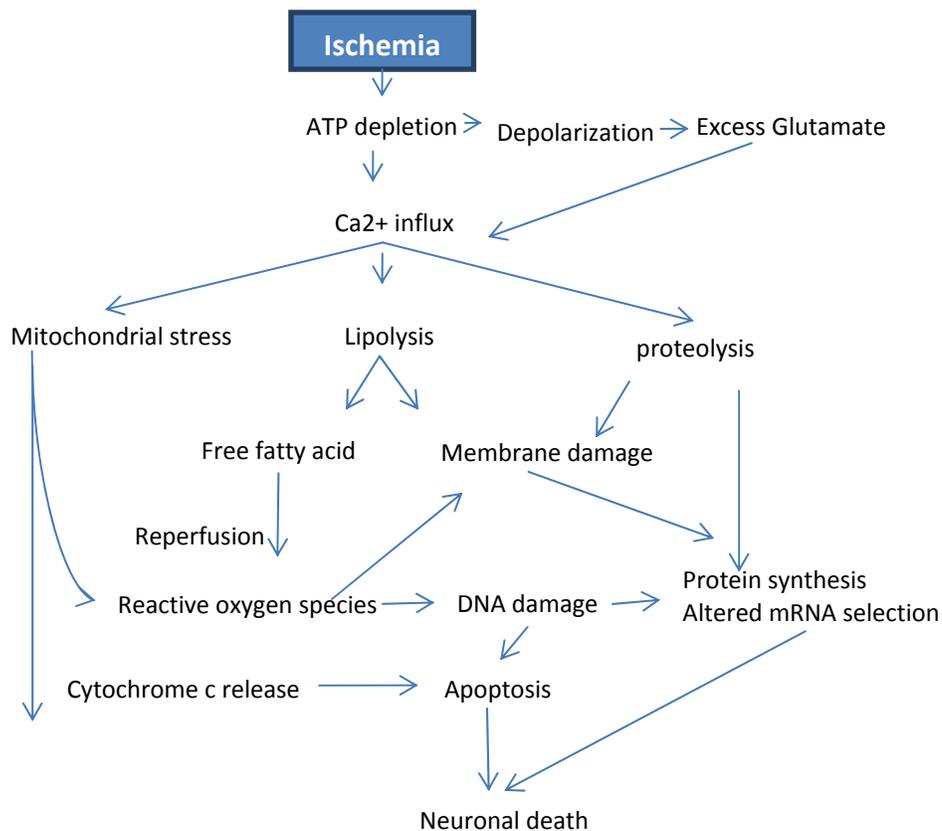


Figure 1-1: Brain ischemic cascade

### 1.1.3 Drug concentration and metabolism in cardiac arrest and heart failure patients

As previously discussed, cardiac arrest and heart failure incident leads to various pathophysiological disturbance in the body including hepatic ischemia, diminished intrinsic hepatic metabolic activity and changes in hepatic blood flow, and can associated with altered drug concentration and metabolism.

Drugs with high extraction ratio such as morphine, lidocaine, and organic nitrates, can be affected by the significantly altered cardiac output and hepatic blood flow. A previous study by

Stenson et al has demonstrated the correlation between decreased cardiac output, hepatic blood flow and increased steady state concentration of lidocaine (Stenson et al., 1971). A previous study has showed the prolonged half-life and metabolites for flolequinan in chronic cardiac failure compared to healthy volunteers, and this may also related to the changes of the flow (Nicholls et al., 1996).

For oral drugs, reduced gastric emptying in heart failure patients delays absorption and decreased the peak plasma concentrations of furosemide, bumetanide and digoxin (Johnston et al, 1992). In addition, after an oral dose, the AUC and half-life of prazosin has doubled in congestive heart failure patients compared with healthy control subjects (Baughman RA et al., 1980). The decreased absorption and first pass metabolism can be offset by reduced hepatic and renal clearance, for oral drugs (Johnston et al, 1992).

Previous study by Hepner et al tested aminopyrine as a probe drug for hepatic metabolic enzyme activity. The study found the significant reduced clearance of aminopyrine in heart failure patients compared with the control ( $29.7 \pm 7.1$  vs  $125.1 \pm 5.7$  ml/min) (Hepner et al., 1978). The prodrugs ACE inhibitors such as enalapril, perindopril, ramipril, generally have lower than expected plasma concentrations in heart failure patients, indicating the potential biotransform activity decreased (Johnston et al, 1992). Quinidine, an antiarrhythmic, is extensively metabolized by the liver. Previous study has shown that in heart failure patients, quinidine concentration was 43% higher and the clearance decreased to 70% of the values in controls (Ueda et al., 1978). In addition, Tokola et al examined liver biopsy of patients of congestive

heart failure and found 30% decrease in the levels of drug oxidizing enzymes as compared to control (Tokola et al., 1973).

The abnormal liver enzymes and liver function in congestive right side heart failure has been long recognized, and often time the acute heart failure is even worse than the chronic. This is thought to be due to direct centrizonal compression and hypoxic damage (Richman et al., 1961; Shibayama Y, 1987; Sokol et al., 2000). The mechanism is due to the fact that the liver gets to fixed amount of cardiac output, the decreases in flow is compensated for by an increase in oxygen extraction leading to anoxic necrosis in the centrizonal area. The increase in venous pressure caused by heart failure leads to atrophy of hepatocytes and causes perisinododial edema, which may lead to decreased oxygen diffusion (Sokol et al., 2000; Dunn et al., 1973). In addition, impairments of left ventricular function can also lead to marked liver enzyme abnormalities (Parisi et al., 1990; Cohen et al., 1974).

Currently, the knowledge of impaired organ function, drug metabolism and distribution in cardiac arrest and other heart failure patients is very limited.

## 1.2 THERAPEUTIC HYPOTHERMIA

### 1.2.1 Therapeutic hypothermia history

The use of hypothermia for clinical purposes has ancient roots, beginning with its usage by the ancient Egyptians, Greeks, and Romans. Hippocrates (460-375 B.C.) advocated packing wounded patients in snow and ice to reduce hemorrhage. In early 1700, people already realized that cold could be used to kill the pain and deep hypothermia could improve the survival rate from various wounds. In the early nineteenth century (1814), Napoleon's Surgeon General Baron Larrey observed that injured soldiers who became hypothermic and were put closer to a fire died more rapidly than those who remained hypothermic in the snow. The concept of hypothermia neuroprotection also dates back to ancient times, with the observation that infants abandoned and exposed to cold often remained viable for prolonged periods (Mordecai et al., 1992; Polderman 2004).

In 1930s, Dr. Temple Fay used therapeutic hypothermia (TH) for 123 cancer patients to help with pain alleviation and to prevent further metastasis. In 1950s, Dr. Bieglow introduced TH to cardiac surgery patients. In 1960s, Dr. Rosomoff and Dr. Safar introduced TH to TBI and other neurological injuries. Most of these studies used relatively deep hypothermia (30 °C or lower). The clinical use of TH in critical care was discontinued for several years because of deep hypothermia's side effects, uncertain benefits and management problems. From the early 1990s, mild to moderate hypothermia (30-35°C) was proven useful for treating focal and global ischemic brain injury in animal models and subsequently re-emerged as a therapeutic

intervention (Fay T, 1938; Alzaga, 2006; Bigelow et al., 1950; Shann F, 2003; Polderman 2004; Safar P, 1994; Safar and Bircher, 1994).

### **1.2.2 Therapeutic hypothermia applications in critical care medicine**

Currently, the use of therapeutic hypothermia between 32-35°C has increased clinically based on the results from randomized controlled clinical trials, which have demonstrated decreased mortality and improved neurological outcomes after cardiac arrest in adults and hypoxic-ischemic encephalopathy (HIE) in neonates.

Due to the low survival rate and high incidence of brain dysfunction for post cardiac arrest patients, hypothermia provided the promising benefit for better neurological recovery and good outcome (Hypothermia after Cardiac Arrest Study Group, 2002; Bernard et al., 2002). Induced hypothermia after successful resuscitation leads to one additional patient with intact neurological outcome for every 6 patients treated (Kochanek and Bakken, 2009). Cardiac arrest patients especially if the initial rhythms of ventricular fibrillation are recommended to receive mild hypothermia at 32-34°C for 12-24 hours post resuscitation by American Heart Association and International Liaison of Committee for Resuscitation since 2003. Until 2009, more than fifty peer reviewed studies were identified investigating mild hypothermia during or after cardiac arrest in human adults. As of early 2011, there are over 200 published studies investigating hypothermia after cardiac arrest in human adult subjects.

Therapeutic hypothermia is a promising therapy for neuroprotection for encephalopathy presumably due to hypoxic ischemia injury (Shankaran et al., 2010; Gluckman et al., 2005;

Thoresen et al., 2005; Polderman and Girbes, 2006). Neonatal HIE is one of the top reasons for infant deaths and brain damage. Neonates with moderate to severe encephalopathy have significant brain function deficits, memory impairment and increased risk of death (Shankaran 2009). A relatively small reduction in temperature from 35°C to 32-30°C hypothermia in neonatal animals was associated with better maintenance of cerebral energy state during or immediately after ischemia (Shankaran 2008). Multiple multi-center clinical trials have consistently demonstrated that hypothermia was associated better outcome for neonatal HIE (Shankaran et al., 2008; Hoehn et al., 2008).

Therapeutic hypothermia is also being evaluated in several other diseases including traumatic brain injury, focal brain ischemia, myocardial ischemia, spinal cord injury, hemorrhagic shock, pediatric cardiac arrest and encephalopathy due to acute liver failure (Schwab et al., 1998; Martinez-Arizala et al., 1992; Heinius et al., 2002; Stravitz, et al., 2009; Linares, et al., 2009; Kochanek et al., 2009).

Therapeutic hypothermia is induced by lowering core body temperature via a wide array of surface cooling and intravenous cooling methods such as fans, cooling blankets, ice pack and intravascular cooling methods. Randomized controlled trials demonstrating neuroprotection have employed therapeutic hypothermia at 32-34°C for 12-24 hours in adults and up to 48-72 hours for pediatric patients (Bernard et al., 2002; Shankaran et al., 2010). Clinical implementation of therapeutic hypothermia can be divided into induction, maintenance and rewarming phases. Generally, rapidly reducing core body temperature, stable and controlled maintenance in the range of 32-35°C, and slow passive rewarming are suggested to be the key variables to yield

beneficial effects. In addition, it is important to identify eligible and ineligible patients and include key people from early in the planning stages to ensure success of therapeutic hypothermia (Oddo et al., 2006; Polderman 2004; Polderman and Herold, 2009; Seder et al., 2009; Thoresen 2011; Callaway 2011).

The success of therapeutic hypothermia in improving neurological outcomes has been attributed to the fact that reduced body temperature alters multiple pathogenic mechanisms simultaneously. Hypothermia inhibits multiple steps in the biochemical cascade that contributes to brain injury after insult including reduce excess glutamate, reduce mitochondrial stress, reduce cytochrome c release, reduce reactive oxygen species release and reperfusion injury, reduce lipolysis, reduce the changes of decreased protein synthesis and altered mRNA selection and positively interacts with postischemic repair processes (Polderman 2009; Shintani et al., 2010; Silasi, et al., 2011, Kochanek et al., 2009; Busto et al., 1989). It is the multiple pathway effects of hypothermia that are believed to underlie the success of therapeutic hypothermia as compared to single pathway pharmacologic agents in producing neuroprotective benefits.

### **1.2.3 Thermoregulation**

Our body temperature has a fundamental influence on nearly all aspects of biological function, including the expression of genes, the activity of enzymes, the rate and force of contraction of muscles, the firing of neurons, and the habitats in which we can live (Blumberg 2002). All warm blooded animals, including humans, have a regulated/controlled body temperature. The normal body temperature for humans is 36.5-37°C. Humans can stay in very cold environments and maintain normal body temperatures. Many mathematical models of thermoregulation in humans

have been developed over the last 50 years (Grahn et al., 2011). Generally, we can consider that human thermal system consisting of core compartment and peripheral compartment. These two compartments regulate each other to adjust with temperature changes in the environment. The core temperature is regulated by limiting or increasing heat transfer to the periphery. Heat loss from the peripheral compartment is regulated through changes in skin perfusion (through vasodilation or vasoconstriction and radiation) and by increasing or decreasing the production of sweat (Polderman 2004).

Our central thermoregulation, is believed to be mainly regulated by preoptic-anterior/hypothalamus (POAH) in the brain, involves the afferent thermo-sensor spinal cord and involves nearly every part of autonomic nervous system (Benarroch 2007; Kurz 2008; Sessler 2009). The normal set point is maintained when all thermoregulatory response are simultaneously turned on or off in response to hypothalamic temperature. Input temperatures outside the normal setting initiate regulatory responses. The core temperature triggering an afferent thermoregulatory response defines the threshold for central thermoregulatory response (Sessler, 2003; Sessler 2007). Body temperature regulation interference interacts with various physiological responses through the autonomic nervous system. In addition, autonomic nervous system is also related to the perception of pain, deep pressure, emotions and metabolic aspects, the endocrine functions as well as chronobiology related activities (Lungu et al., 1966; Haus 2007).

In many clinical situations, including disease states and drug therapies, the central set point for thermoregulation can be interrupted. General anesthesia markedly impairs normal control of

body temperature, reducing the threshold (triggering core temperature) for thermoregulatory vasoconstriction from 37°C to 34.5°C. Local anesthesia can also decrease the thresholds triggering vasoconstriction and shivering approximately 0.6°C (Sessler 2008; Sessler 2009). Consequently, most anesthesiologists consider mild hypothermia therapeutic during procedures (such as neurosurgery) in which cerebral ischemia is likely. In addition, sedatives,  $\mu$ -opioid antagonist, biogenic amines, 5-hydroxytryptamine (5-HT), norepinephrine,  $\alpha$ -adrenoceptor agonist, dopamine receptor antagonist and NMDA receptor antagonist all have been reported to affect thermoregulatory set point (Weant et al., 2010; Arpino et al., 2008; Chamorro et al., 2010). On the other hand, inflammation can cause hyperthermia, which most of time is also considered as a protective mechanism. Other physiological and pathological brain hyperthermia has been discussed in Kiyatkin, 2007.

#### **1.2.4 Physiological effect of therapeutic hypothermia**

Therapeutic hypothermia is a clinical based, forced, artificial cooling. In this thesis research the work was focused on the clinically relevant temperature range from 32-35°C. Homeostatic mechanisms act to counter forced reductions in body temperature during therapeutic hypothermia and produce a series of physiological stress responses as well as cellular and organ function changes. These observed physiological responses can be temperature specific and time specific (Table 1-1).

##### ***1.2.4.1 Thermoregulatory response***

The normal physiological response to hypothermia onset is peripheral vasoconstriction to preserve core normothermia (Warner 2009). Shivering often happens at the period of

hypothermia induction when body attempts to generate heat to counteract the cooling. A frequently cited threshold for shivering is 35.5°C. Shivering increases the total body metabolic rate by 2-4 fold, therefore raise concerns for myocardial stress in patients with vascular disease and hypothermia induction efficiency (Warner 2009). Shivering is readily inhibited with neuromuscular blockers, and combination of opioids, sedative, general anesthetics and may require mechanical ventilation (Doufas et al., 2004; Doufas 2003).

#### ***1.2.4.2 Cardiovascular system***

Hypothermia induction may trigger the thermoregulatory and cardiovascular response such as shivering and increased heart rate. A core temperature reduction of 1°C in normal volunteers is associated with 20% increase in myocardial perfusion and 33% increase in heart rate, which is predicted parallel by increases in plasma norepinephrine and epinephrine (Frank et al., 2003; Warner 2009). Normally, tachycardia is observed when temperatures are above 35°C and bradycardia when temperatures are below 35°C (Polderman 2004). Mild hypothermia at temperatures below 35°C, decreased heart rate and the slowed metabolic demand accompanied a decrease in cardiac output of about 25% (Polderman 2004). Hypothermia is associated with changes in myocardial function. The cardiac index is transiently suppressed while systemic vascular resistance is transiently increased during hypothermia induction. These changes spontaneously correct by 6-18 hours after hypothermia onset (Bernard et al., 2002; Warner 2009). In addition, hypothermia induced arrhythmia may occur for temperatures lower than 30°C.

#### ***1.2.4.3 Blood system***

Watts et al. showed the slowed enzyme activity and decreased platelet function contributing to hypothermic coagulopathy in patients when temperature below 34.0°C. There was no change in fibrinolysis within the studied temperature range from 33-36°C (Watts et al., 1998). Lower pH has been found during hypothermia as the solubility of CO<sub>2</sub> in blood increases at lower temperatures. In addition, lactic acid levels increase with shivering during hypothermia. A previous study also indicated that hypothermia and acidosis impair thrombin generation and fibrinogen availability (Martini 2009). Future study in this area is still needed for confirmation.

#### ***1.2.4.4 Renal function***

As measured by creatinine clearance, renal function is impaired during hypothermia, but this appears to be reversible after rewarming (Arpino 2008). In a previous review paper, van den Broek et al. proposed that if the synthesis of creatinine decreases, the renal function may not be well predicted by creatinine clearance (Broek et al., 2010). Urine output usually increases in mild hypothermia initially, due to an increase in renal blood flow of vasoconstriction. Then with falling temperature, the increase in urine output may due to a loss of distal tubular ability to reabsorb water and a resistance to action of vasopressin (Polderman 2004; Mallet 2002). The cold induced diuresis is accompanied by an increase in urinary electrolyte excretion, probably as a result of reduced tubular sodium re-absorption (Atterhog 1975). Serum levels of K<sup>+</sup>, Mg<sup>2+</sup>, Ca<sup>2+</sup> and phosphorus are significantly reduced during hypothermia. Compared with other organs, renal oxygen consumption is most rapidly reduced in hypothermia (Wong 1983). Renal blood flow reducing during hypothermia has been suggested, and it may be attributed to an increase in renal vascular resistance or decreased cardiac output, which may also related to specific

temperature range (Wong 1983). Previous study by Nishida et al. observed that GFR (glomerular filtration rate) did not decrease until the core temperature was below 32°C (Nishida et al., 2007). This study suggests that kidney is able to maintain GFR in spite of possible variations in the systemic circulation.

#### ***1.2.4.5 Hepatic and metabolic***

The liver's functions of detoxification and conjugation are believed to be depressed during hypothermia, therefore, affecting the half-life of many drugs, and prolonged effect of ethanol (Wong 1983). Liver enzyme activities have been shown to be temperature dependent; therefore decreases in activity are predicted during systemic hypothermia (Wong 1983). Oxygen consumption is reduced about 6% for every degree C fall in temperature indicating the general metabolism rate is reduced in parallel. The basal metabolic rate can be reduced by as much as 50% at 28°C. Cardiac arrest injury related hepatic ischemia might also affect liver metabolism. Hepatic metabolism decrease coupled with decreased renal blood flow will result in decreased drug clearance depending on the degree to which the given drug is extracted by liver enzymes. These alterations in liver enzyme activity can lead to overtreatment or delayed awakening for anesthesia drugs (Diaz, 2011).

#### ***1.2.4.6 Gastrointestinal***

Intestinal motility decreases below 34°C. The absorption of medication given orally or by nasogastric tube can be impaired during hypothermia (Polderman 2004; Mallet 2002). The gastric erosions and submucosal hemorrhages have found in hypothermia patients, although the authors suggested it was not clinical significant (Reuler 1978).

#### **1.2.4.7 Others**

Mild hypothermia suppresses insulin release and cause insulin resistance. Peripheral uptake of insulin at the tissues is impaired (Polderman and Herold 2009; Polderman 2009). Possible airway infections, wound infections and prolonged healing were observed (Polderman 2004). Hypothermia also influences the immune system, which has not yet proven to contribute to hypothermia neuroprotection, but it may relate to the increases the risks of infection. More studies are needed for conclusion (Polderman 2004; Meybohm et al., 2009; Scumpia et al., 2004). These physiology changes might be time dependent, with forced cooling change to regulated hypothermia, the body will tend to adapt the environment with adjusted physiology parameters. In addition, a series of complex physiological changes will be observed under a combination of disease injury and hypothermia effect.

#### **1.2.5 Rewarming**

Rewarming, normally using a heating air blanket is initiated after the recommended period of induced therapeutic hypothermia, to increase body temperature back to normothermia. Rewarming is one of the important variables in the therapeutic hypothermia. Physiological effects and cerebral effects of rewarming following a prolonged hypothermia are not fully understood. Most studies suggested a slow rewarming is most beneficial. In the cases of which intracranial hypertension is controlled only with hypothermia, a slower rewarming period should be established (Varon 2008). Although the optional rewarming rates remain to be determined, it is likely depends on the disease process and the presence or absence of brain swelling (Kochanek, 2009). Comparing literature reported rewarming rate in different patients population, the fastest rewarming rates are used in cardiac arrest patients (no faster than 0.5°C per hour),

slower rewarming rates are generally used in TBI (no faster than 0.25°C per hour) and even slower are used in stroke patients (Kochanek, 2009).

Therapeutic hypothermia over the range of 32-35°C affects multiple physiologic processes including reduced metabolic rate, changes in pH and renal function, bradycardia and slowed gastrointestinal function and so on. Each of these physiological processes are also involved in determining a given drugs dispositional characteristics (Table 1-1). It is this fact that led us to propose that TH would have a significant effect on drug disposition and effect.

Table 1-1: The effect of hypothermia on major organ systems

Organ system	Hypothermia effect	Affected drug group
Cardiovascular	flow↓ in disease state, hypertension/hypotension, tachycardia >35°C, bradycardia <35°C, CO↓ <35°C	High hepatic extraction drug
Renal	diuresis, vascular resistance↑, <35°C vascular resistance↑, electrolytes disorder, renal oxygen consumption↓, tubular dysfunction	Renal secretion drug, hydrophilic drugs
Hepatic	<35°C flow↓, liver enzyme activity ↓, liver function impairment in disease	Liver enzyme dependent drugs
Gastrointestinal	Intestinal motility↓, Gastrointestinal impairment	Orally given drugs
Metabolic	Metabolic demand↓, CO <sub>2</sub> production↓, O <sub>2</sub> consumption↓	
Blood	Platelet system interrupted<34°C	

### 1.3 THE EFFECTS OF HYPOTHERMIA ON DRUG METABOLISM AND DRUG RESPONSE

Zhou J and Poloyac SM The effect of therapeutic hypothermia on drug metabolism and response: cellular mechanism to organ function. *Expert Opinion of Drug Metabolism and Toxicity*, 2011;7(7):1-14.

The focus of this thesis is to determine the effect of hypothermia and cardiac arrest on drug metabolism. As previously discussed many of the enzymes and transporters involved in drug absorption, distribution and metabolism are temperature dependent. Therapeutic hypothermia has been utilized clinically without a full understanding of the effects on drug metabolism and drug disposition. The extensive pharmaco-therapeutic regimen employed in the critically ill patients, coupled with the difficulty identifying adverse drug reactions in these patients, creates a high likelihood of unrecognized therapy–drug interactions. For clinicians and health professionals to better understand the best method of applying hypothermia during pharmacological management, an understanding of the effect of hypothermia on current knowledge of drug disposition and response is necessary. In this chapter, a literature review of previous preclinical and clinical evidence and potential implications of altered drug disposition during mild and moderate hypothermia (30-35°C) is presented.

#### **1.3.1 Pharmacological interventions involved in therapeutic hypothermia**

A wide variety of medications are employed in critically ill patients who receive therapeutic hypothermia. These medications include analgesics/sedatives, muscle relaxant, cardiovascular

drugs, anti-arrhythmics, anti-hypertensive, anti-convulsant, anti-platelets, antimicrobial and anti-inflammatory drugs (Arpino et al., 2008; Chamorro et al., 2010). We summarized the list of these medications in Table 1-2 with their pharmacological effects, related specific metabolic and elimination pathways, liver extraction ratios, protein binding and half-lives (Chernow, 1989; Kim 2002; Kalliokoski 2009). Many of these drugs have narrow therapeutic windows and have not been thoroughly studied under conditions of therapeutic hypothermia. Adverse drug effects in critically ill patients are known to cause hypotension, increased ICU stay, prolonged cardiovascular support, prolonged respiratory depression and hematologic side effect (Devlin et al., 2010; Lazarou et al., 1998). These adverse effects have the potential to diminish the overall efficacy of therapeutic hypothermia.

Table 1-2: Pharmacological interventions involved in critically ill patients and their pharmacokinetic characteristics

<b>ANALGESICS/SEDATIVE</b>	Half life	Protein binding	Hepatic extraction ratio to blood flow	CYP3A	CYP2C9/19	CYP2D6	Renal elimination	Transporter/ OTHERS
Fentanyl	2.5-6.5mins	80-85%	High	√			√	
Propofol	30-60mins	95-99%	High				√	CYP2B/UGT
Alfentanil	1.5-2 hrs	92%	Low	√			√	
Midazolam	1.8-6.4hrs	95%	Low/Intermediate	√			√	
Morphine	2-3hrs	30-40%	High				√	UGT
<b>MUSCLE RELAXANT</b>								
Vecuronium	51-80mins	30%	Low	√			√	PGP
Pancuronium	1.5-2.7hrs	77-91%	Low				√ +Bile	
<b>ANTI-ARRYTHMICS</b>								
Lidocaine	1.5-2hrs	61-80%	High	√			√	CYP1A2
Amiodarone	15-142 days	33-65%	Low		√		Bile	
Digoxin	38-48hrs	25%	Low				√	PGP
Procainamide	2.5-4.5hrs	15-20%	Intermediate			√	√	
Bretylium	7-8hrs	N/A	N/A				√ only	
<b>ANTI-HYPERTENSIVE</b>								
Verapamil	2.8-7.4hrs	90%	Intermediate	√	√			PGP
Enalapril	11hrs	N/A	Intermediate				√	OATP/ MRP2
Metoprolol	3-7hrs	15%	Intermediate/high			√	√	
Propranolol	4-5hrs	88%	High			√		
Atenolol	6-7hrs	6-16%	Low				√	
Valsartan	6hrs	95%	N/A				√+Bile	OATP/MRP2
Pravastatin	77hrs	50%	Intermediate/high				√	OATP1B1 / OATP2B1
<b>RESUSCITATION/ INCREASE HEART RATE</b>								
Epinephrine	2mins	N/A	N/A				√	MAO/COMT
Dopamine	9mins	N/A	N/A					MAO/COMT
<b>ANTI-CONVULSANT</b>								
Phenytoin	6-24hrs	90%	Intermediate		√		√+Bile	UGT
Phenobarbital	2-7 days	20-45%	N/A		√		√	CYP2C19/UGT
Carbamazepine	25-65hrs	76%	Low		√			PGP/UGT
<b>MISCELLANEOUS</b>								
Warfarin	30-60hrs	99.5%	Low		√ (S-,R-)		√	CYP1A2(R-)
Mannitol	100mins	N/A	N/A				√ only	
Gentamicin	2 hrs	0-10%	N/A				√	
Rifampin	1.5-5hrs	90-95%	N/A				√	Esterases
Ranitidine	2-3hrs	15%	N/A			√	√	PGP
Famotidine	8-12hrs	10-28%	N/A				√	
Corticosteroids	Varies	varies	N/A	√			√	
Magnesium Sulfate	N/A	NA	N/A				√ only	

### **1.3.2 Metabolism and hepatic clearance alterations during therapeutic hypothermia**

In this section, we reviewed the current literature on the alterations in either drug concentrations or metabolism during hypothermia within the temperature range 30-35°C. It has been shown that hypothermia increases drug concentration and prolongs drug response in multiple clinical and animal studies. Based on the classic Pang et al. paper describing the well-stirred model (Pang and Rowland, 1977), factors that affect the hepatic clearance of a drug include: 1) blood flow to the liver; 2) intrinsic clearance; and 3) the fraction of drug bound to protein. Drugs can be classified into three categories according to the well-stirred model equation  $CL_h = Q \times [F_u \times CL'_{int} / (Q + F_u \times CL'_{int})]$ : 1) Flow limited: in the case when intrinsic clearance ( $CL'_{int}$ ) is very large in comparison to blood flow ( $Q$ ), hepatic clearance ( $CL_h$ ) proximately equals blood flow,  $CL_h = Q$ ; 2) Capacity limited binding sensitive: for drug highly bound to plasma protein, a displacement from binding sites will significantly increase the concentration of drug at the hepatic enzyme site and the rate of metabolism will increase,  $CL_h = F_u \times CL'_{int}$ ; 3) Capacity limited binding insensitive: When  $CL'_{int}$  is very small comparison to hepatic blood flow, assigning drug protein binding did not change during the therapeutic range,  $CL = CL'_{int}$ . Therefore, in this section, we divided all the drugs into three categories based on the flow limited/hepatic extraction ratio, protein binding and their specific metabolic pathway (Table 1-3).

#### ***1.3.2.1 Flow limited drugs in therapeutic hypothermia***

Flow limited drugs have a high hepatic extraction to blood flow ratio. Upon i.v. administration, the systemic clearance is highly dependent on the liver blood flow. Propofol and fentanyl are two examples of flow-limited drugs. During hypothermia at 34°C, propofol steady state concentrations during constant infusion increased 28% compared with normothermia at 37°C in

human volunteers ( $p < 0.05$ ). In addition, the effect of hypothermia on propofol concentration was more significant in the beginning ( $3.34 \pm 1.62$  vs  $1.99 \pm 1.30$  pg/ml at 2 min), with diminishing magnitude of change at later time points. The three-compartment model was used to estimate the pharmacokinetic parameters of propofol. The clearance of propofol at  $34^{\circ}\text{C}$  decreased 25% compared with normothermic condition ( $0.59$  (95% CI 0.24 - 1.38) vs  $0.79$  (0.58 - 1.08) L/min). A significant association was found between hypothermia and the inter-compartment clearances ( $1.73$  (95% CI 1.40 - 2.15) vs  $3.51$  (3.40 - 3.63) L/h for shallow,  $p < 0.005$ , and  $2.13$  (95% CI 1.83 - 2.47) vs  $2.63$  (2.15 - 3.22) L/h for deep,  $p < 0.05$ ). Hepatic blood flow decreased in both groups ( $33 \pm 11\%$  in hypothermic volunteers and  $23 \pm 11\%$  in normothermic group); however, this trend did not reach statistical significance (Leslie et al., 1995).

Similarly, in an animal study, Fritz et al. showed that fentanyl plasma concentrations are significantly elevated by  $25 \pm 11\%$  in  $31.6^{\circ}\text{C}$  as compared with normothermic group in pig model. Fentanyl was given infusion at a constant rate during a 6-hour period of hypothermia at  $31.6 \pm 0.3^{\circ}\text{C}$ . Fentanyl plasma concentration at the end of the cooling was  $7.0 \pm 0.6$  ng/mL, which was significantly elevated as compared with normothermic group ( $< 6.0$  ng/mL). The authors suggested that the porcine model has similarities between swine and humans in the basic cardiovascular variables, as well as regional distribution of blood flows. Significant reduction in cardiac output and reduced flow to multiple organs were found during hypothermia. However, there was no significant alteration in hepatic artery flow during hypothermia (Fritz et al., 2005).

Meanwhile, in two animal studies, the metabolism and elimination of indocyanine green (ICG), a commonly used marker for liver function, especially hepatic blood flow and biliary excretion has been tested in hypothermia. Daemen et al. has shown that the plasma disappearance ICG was delayed in hypothermia 32.5°C and the clearance of ICG was significantly reduced from normothermia in male Wistar rats (Daemen et al., 1986). Pentobarbitone anesthesia was used in the study. ICG clearance decreased in the normothermia group as well ( $4.3 \pm 0.6$  vs  $8.0 \pm 1.7$  ml/min in conscious rats). Hypothermia further reduced the clearance of ICG to  $0.9 \pm 0.1$  ml/min, which was almost an 80% decrease from normothermic group clearance. Mean resident time (MRT) increased two-fold in hypothermia. In addition, Nishida et al. has shown that during hypothermia at 32°C, significantly elevated ICG plasma concentration, increased area under the curve (AUC), and the decreased total clearance of ICG were found (Nishida et al., 2007). ICG was given IV at 1mg, plasma AUC at hypothermic group increased approximately two-fold compared to the AUC from control group ( $550 \pm 63.3$  vs  $271.4 \pm 53.4$   $\mu\text{g} \times \text{min/mL}$ ). Total clearance of ICG in hypothermic group decreased to 47% compared to those in normothermia ( $2.28 \pm 0.26$  vs  $4.83 \pm 0.70$  ml/min).

Based on these results, it is evident that the clearance of the high extraction ratio drugs, propofol and fentanyl are significantly reduced during hypothermia. Although this alteration is likely due to a reduction in hepatic blood flow, inconsistent results have been published demonstrating either no change or a decrease in hepatic blood flow during cooling. These inconsistent changes in blood flow may due to the different assessment methods. In addition, at specific time or specific temperature, blood flow pattern compared to normothermia might be different. Mild hypothermia initially may have no effect on blood flow, but with sustained low temperature

during prolonged cooling, reduced blood flow is more likely observed due to a lower metabolic demand. Definitive studies employing more precise experimental methods of assessing hepatic blood flow are needed for full elucidating the mechanisms that underlie changes in fentanyl or propofol clearance.

### ***1.3.2.2 Capacity limited binding sensitive drugs in therapeutic hypothermia***

Phenytoin is an example of capacity limited binding sensitive drugs. It is metabolized through CYP2C9 and CYP2C19 isoforms. Phenytoin metabolism and protein binding have been tested in mild hypothermia in the study by Iida et al. in patients (Iida et al., 2001). Fourteen traumatic brain injury patients were cooled to 34°C for 72 hours. Phenytoin AUC was increased by 180% ( $46.3 \pm 30.2$  vs  $16.6 \pm 7.3$  min·mg/mL,  $p < 0.02$ ) and mean residence time was prolonged by 180% ( $194 \pm 130$  vs  $69 \pm 28$  minutes,  $p < 0.01$ ) during hypothermia compared with the corresponding values after rewarming. The clearance of phenytoin was decreased by 67% ( $1.11 \pm 0.86$  vs  $3.42 \pm 2.67$  L/h,  $p < 0.02$ ) and elimination constant ( $K_e$ ) was decreased by 50% ( $0.10 \pm 0.06$  vs  $0.20 \pm 0.15$  h<sup>-1</sup>,  $p < 0.05$ ) during hypothermia. In addition, plasma concentration of metabolite 5-pHPPH was found to be significantly lower during hypothermia in comparison with after hypothermia ( $p < 0.05$ ). Phenytoin protein binding did not change during hypothermia. Therefore, these results demonstrated that phenytoin metabolism was inhibited by mild therapeutic hypothermia in the study, whereas protein binding was not altered, thereby implying that hypothermia decreased the specific activities of CYP2C9 and CYP2C19.

In a cardiac arrest animal model, our lab has shown that protein binding for chlorzoxazone at 30°C did not change from normothermia values (Tortorici et al., 2006). A confounder in

assessment of protein binding was discussed by van den Broek et al. who has pointed that the in vitro protein binding test might not be accurate because hypothermic blood has been warmed up to 37°C. Therefore, the drug protein binding formed a new equilibrium before the analysis was carried out, which would introduce errors in predicting the temperature effect (van den Broek et al., 2010). However, in our lab previous observation with chlorzoxazone, careful temperature control was conducted throughout experimentation to decrease the likelihood of this confounder. Lin et al. has suggested that use of the ratio of cerebrospinal fluid (CSF) drug concentration to plasma drug concentration to determine the drug binding in vivo, since CSF is a very low-protein fluid, drug in CSF is considered to be primarily unbound (Lin et al., 1997). Future studies providing available data from both blood and CSF concentrations during hypothermia will be helpful in further evaluating the protein binding change in vivo.

Collectively, the current evidence suggests that mild to moderate hypothermia does not significantly alter drug protein binding. However this conclusion is based on studies in a limited number of drugs, therefore future research is needed to determine the magnitude of hypothermia effect on protein binding across substrates.

### ***1.3.2.3 Capacity limited binding insensitive drugs in therapeutic hypothermia***

The clearance of capacity limited drugs is highly dependent on the hepatic intrinsic clearance, including, hepatic drug metabolism by CYP enzymes. Many drugs used in critical care medicine are metabolized by various isoforms of the CYP450 enzyme (Table 1-1). In this section, we reviewed the published evidence on evaluating drug concentration and pharmacokinetic

alterations. Individual drugs have been divided by their specific metabolic CYP pathway (Table 1-3).

#### *1.3.2.3.1 CYP3A*

CYP3A is one of the most important CYP isoforms. CYP3A has broad substrate specificity, metabolizing a wide array of compounds such as the small molecules acetaminophen through the large molecule such as cyclosporine. A typical substrate structure includes lipophilic, 1-2 H-bond donor/acceptors at 5.5-7.5Å and 8-10Å from the binding site (Kumar et al., 2001). Commonly used drugs in critical care metabolized by CYP3A include midazolam, fentanyl, alfentanil, cardiovascular drugs such as lidocaine, calcium channel blockers vecuronium and the majority of corticosteroids. Since CYP3A is present in both liver and intestine, the changes in activity of CYP3A in the intestine could also affect both bioavailability and systemic clearance.

Midazolam is a well-known CYP3A4/5 substrate. A previous study by Fukuoka et al. has reported a five-fold increase in plasma concentration of midazolam in hypothermic patients compared with the normothermic patients at steady state (Fukuoka et al., 2004). The highest concentration was found at 96 hours which was close to 10,000ng/mL in hypothermic group, while the concentration at steady state in normothermic group was less than 2000ng/mL. The hypothermic patients were maintained for 48 hours and slowly rewarmed. The complete sample collection time was 216 hours after ICU administration. There was greater than 100-fold decreases in clearance of midazolam in these brain injured patients when subjects' core temperature were lower than 35°C. In addition, the calculated volume of distribution in hypothermia was 84% higher than normothermia. In later part of this thesis, we will discuss one

of our human studies in Chapter 4. In this study, we evaluated midazolam clearance in normal healthy volunteers during mild hypothermia. We found that the midazolam clearance and inter-compartment clearance were associated with temperature reduction. Systemic clearance of midazolam has been estimated to decrease 11.1% per °C reduction in body temperature (Hostler et al., 2010). Therefore, the significant increases in midazolam concentration and decreased clearance observed in the studies are likely due to the depressed CYP3A intrinsic clearance. Similarly, neuromuscular blocker vecuronium is metabolized by CYP3A and the clearance of vecuronium was estimated to decrease 11.3% per °C in healthy human volunteers by Caldwell et al. (Caldwell et al., 2000). The percentage alteration in plasma clearance per °C reduction in body temperature can be very helpful in developing dosing guidelines in patients receiving therapeutic hypothermia.

The decreased CYP3A activity in vitro has been reported by Fritz et al., in which ethyl morphine-N-demethylation experiment showed strong temperature dependence of CYP3A activity ( $p < 0.01$ ) (Fritz et al., 2005). Hepatic CYP3A4 activity decreased to  $69 \pm 1\%$  at  $32^\circ\text{C}$  when compared with the CYP3A4 activity in normothermic control ( $p < 0.001$ ). Future in vitro study testing different concentrations of substrate as well as different temperatures will be helpful.

Collectively, these studies suggest that the metabolism of the drugs that depend on CYP3A activity would be inhibited due to the acute effect of hypothermia on CYP3A functional activity. The magnitude of the alteration is still open for debate given the data suggesting from 100-fold to as little as 20% changes during mild to moderate hypothermia. Future studies are warranted to determine the magnitude and clinical implications of these changes in CYP3A function.

#### 1.3.2.3.2 CYP2C9 and CYP2C19

The CYP2C9 and CYP2C19 isoforms are involved in the metabolism of many commonly used medications in critically ill patients. Typical CYP2C9 substrate characteristics are weak acid, lipophilic, 1 or 2 H-bond donor/acceptors at 5-8Å from the site of metabolism. CYP2C9 also displays polymorphism in humans (Kumar et al., 2001; Stearns et al., 1995). CYP2C9 metabolizes drugs including phenytoin, carbamazepine, warfarin, tolbutamide, neostigmine and losartan. Typical substrates of CYP2C19 are neutral or weakly basic, 2-3 H-bond done/acceptors at 4.5Å apart and 5-8Å from the site. CYP2C19 is also involved in metabolizing phenytoin, phenobarbital and carbamazepine. In addition, it metabolizes omeprazole, diazepam and propranolol.

The metabolism of phenytoin has been discussed in the previous section, in which significant metabolism inhibition during hypothermia has been reported (Iida et al., 2001). Neostigmine is metabolized by CYP2C9 and CYP3A. Heier et al. demonstrated that at normothermia condition, the clearance and central volume of distribution of neostigmine were  $696 \pm (\text{SE } 175)$  ml/min and  $5590 \pm (\text{SE } 926)$  ml in healthy human volunteers. During mild hypothermia at 34.5°C, there was a 38% reduction in the central volume of distribution of neostigmine, and no significant alteration of total clearance was found (Heier et al., 2002). In this case, the reduction of volume of distribution can describe the plasma concentration-time profile better than the reduction of clearance. Acenocoumarol is also metabolized by CYP2C9. Acenocoumarol is an anticoagulant that functions as a vitamin K antagonist. In the animal study, clearance of S-acenocoumarol decreased significantly during hypothermia at 32.5°C in male Wistar rats (Daemen et al., 1986). At normothermia 37.5 °C, pentobarbitone anesthesia decreased clearance of acenocoumarol from

those in conscious rat ( $4.9 \pm (\text{SE } 0.4)$  vs  $3.4 \pm (\text{SE } 0.3)$  ml/min). Hypothermia further reduced the clearance to  $2.5 \pm 0.2$  ml/min, which was around 49% decrease from normothermia conscious state rats and 26.5% decrease from normothermia anesthesia group.

Phenobarbitone is metabolized by CYP2C19. Kadar et al. performed a study in critically injured children and found that the reduction in body temperature influenced the rate of metabolism, urinary excretion and the volume of distribution of phenobarbitone (Kadar et al., 1982). Urine excretion for metabolite hydroxyphenobarbital at hypothermia 30-31°C was significantly decreased to 48% ( $1.87 \pm 0.71$  vs  $4.50 \pm 0.90$   $\mu\text{mol/h}$ ,  $p < 0.005$ ) while the urinary excretion rate of the parent drug was 52% higher ( $12.01 \pm 3.38$  vs  $6.25 \pm 1.25$   $\mu\text{mol/h}$ ,  $p < 0.05$ ) during hypothermia. Volume of distribution increased around 25% in hypothermia. Relatively large inter-individual variability was observed in the study; however the change in the relative concentration of phenobarbitone and metabolites in urine was influenced primarily by body temperature. Collectively, these data suggest that hypothermia likely decreases the activity of CYP2C19 and rate of metabolism of its substrates.

Collectively, the metabolism and distribution for CYP2C9 and CYP2C19 substrates phenytoin, S-acenocoumaril and phenobarbitone were significantly altered during hypothermia. Clinically, based on the narrow therapeutic window for anticoagulants and anticonvulsant, carefully clinical drug monitoring is warranted for these drugs during hypothermia.

#### *1.3.2.3.3 CYP2D6*

While CYP2D6 constitutes only about 2% of the total CYP content, it is an important isoform due to its role in the metabolism of a large number of pharmaceutical agents such as antiarrhythmics, CNS agents, codeine and anticancer drugs. CYP2D6 also exhibits genetic polymorphisms in humans. While CYP3A4 can accommodate molecules that are structurally very diverse, CYP2D6 has a very strict structure requirement. Substrate normally have at least a basic nitrogen, have a flat hydrophobic area near the site of oxidation, be relatively hydrophilic, and have two potential hydrogen bonding groups in order to bind to the CYP2D6 active site (Kumar et al., 2001).

Biotransformation of pentobarbital involves CYP2D6 and CYP2B6. The pharmacokinetics of pentobarbital during hypothermia (<32°C) was examined in 11 children with Reye syndrome, hypoxic encephalopathy, or acute head injury. A significant decrease in the systemic clearance and a 20% decrease in volume of distribution at steady state of pentobarbital were observed in hypothermic patients when compared to previous data in normothermic adult volunteers (Schaible et al., 1982). The diminished systemic clearance of pentobarbital may result from the decrease in intrinsic enzyme activity that accompanies hypothermia, as well as hepatic dysfunction in patients with Reye syndrome, hypoxic encephalopathy, or acute head injury. In the animal model, the metabolism of pentobarbital-2-C14 during hypothermia at 30°C was studied by Kalser et al. (Kalser et al., 1968). Since liver is the only site of metabolism of pentobarbital, the disappearance of the unchanged pentobarbital from the blood, after initial uptake by the liver, is primarily due to its liver metabolism. This study showed that the hepatic clearance decreased to 50% and the metabolite concentration in blood decreased 32% during

hypothermia. In addition, bile clearance of pentobarbital decreased 71% and less metabolite excreted in bile as well. Therefore, hypothermia markedly decreased the amount of metabolites appearing in blood and bile as well as liver itself.

Rocuronium is partially metabolized through CYP2D6 and CYP2C19 and large amount of rocuronium is excreted unchanged in bile and urine. Beaufort et al. study showed that hypothermia at  $30.4 \pm 0.8^\circ\text{C}$  reduced the plasma clearance of rocuronium around 50% (from  $4.26 \pm 0.50$  to  $2.17 \pm 0.62$  mL/ kg/ min), and prolonged the mean residence time approximately 1.9-fold (from  $56 \pm 19$  to  $108 \pm 39$  min) in nineteen neurosurgical patients (Beaufort et al., 1995). The significant reduction in the clearance of pentobarbital and rocuronium are likely due to the reduction in the metabolic rate. Studies designed to determine the effect of hypothermia on the metabolism and pharmacokinetics of other CYP2D6 substrate drugs are needed to support these results for further conclusion on the effect of hypothermia on the activity of CYP2D6 isoform.

#### *1.3.2.3.4 CYP2E1*

CYP2E1 comprises 7% of total P450 content. It is implicated in the metabolism of volatile anesthetics, acetaminophen, ethanol, and a small number of therapeutic agents. *CYP2E1* active site is relatively restricted compared to the large and open bonding pocket of CYP3A4. The active site contains two phenylalanines at alignment position 78 and 88 which appears to be able to adopt a coplanar arrangement of the phenyl rings, possibility represent an access channel for planar aromatic substrates (Carrière et al., 1996). In addition, humans exhibit wide inter-individual variability in concentrations of CYP2E1 mRNA and protein. The CYP2E1 activity ranges four to five- fold differences from different individuals.

Chlorzoxazone is a well-known CYP2E1 probe. Rate of chlorzoxazone-6-hydroxylation was significantly correlated with concentrations of immunochemically measured CYP2E1 level (Carrière et al., 1996). Our laboratory demonstrated that hypothermia at 30°C decreased the systemic clearance of chlorzoxazone in cardiac arrest rats ( $1.26 \pm 0.34$  vs  $0.580 \pm 0.37$  mL/min). Significant decreases in 6-hydroxychlorzoxone formation clearance and increases in plasma AUC of chlorzoxazone were found in temperature at 30°C compared to normothermia at 37°C (Tortorici et al., 2006). In vitro analysis demonstrated that Michaelis-Menten constant ( $K_m$ ) was significantly increased at 30°C compared to 37°C ( $551 \pm 150$  vs  $255 \pm 52$   $\mu$ M), and CYP2E1 enzyme capacity ( $V_{max}$ ) was not altered. It was concluded that these alterations were due to the reduced intrinsic metabolic clearance produced by a decrease in enzyme substrate affinity and were not due to the alteration in drug protein binding.

Despite the limited number of studies, the results have consistently demonstrated the reduced intrinsic clearance and depressed enzyme activity, including multiple CYP isoforms during mild and moderate hypothermia.

### **1.3.3 Phase II enzymes function in therapeutic hypothermia**

The activities of Phase II enzymes are important for drug biotransformation and elimination. UDP-glucuronosyltransferase (UGT) is one of the most studied phase II enzymes. Endogenous substrates of UGT enzymes include bilirubin, thyroxin, and steroid hormones. Commonly known UGT substrate drugs include benzodiazepines, morphine, propofol, phenobarbital, propranolol, aspirin, acetaminophen and valproic acid (Kumar et al., 2001).

Morphine is primarily metabolized by UGT2B7, with little to no contribution by Phase I enzyme in the morphine elimination. Two major metabolites are morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). M6G binds to  $\mu$ -receptors and is a more potent analgesic than morphine (Wittwer et al., 2006). The effect of mild hypothermia at 33-34°C on morphine concentration in infants with hypoxic-ischemic encephalopathy was studied by Róka et al. (Róka et al., 2008). The study found that the mean morphine concentration in hypothermia was significant higher than normothermic group at 72 hours after birth. More notably, 6 of 7 infants in the hypothermia group vs 1 of 6 infant in the normothermia group demonstrated a concentration above the 300ng/mL. The clearance of morphine in hypothermia was significantly decreased from 0.89 (0.65-1.33) to 0.69 (0.58-1.12) mL/min/kg. The results showed that plasma morphine concentration were more likely to be elevated to potentially toxic concentrations in HIE patients receiving therapeutic hypothermia.

In the animal model, a previous study by Bansinath et al. showed a significantly higher concentration of morphine in both plasma and in cerebrospinal fluid (CSF) during hypothermia (30°C) in dogs (Bansinath et al., 1998). Morphine sulfate was given IV bolus at dose 1mg/kg. Total clearance and volume of distribution of morphine were significantly decreased in hypothermia (25±4 vs 84±11 ml/min/kg, and 5.7±0.8 vs 8.5±0.7 L/kg, respectively) compared to those in normothermia at 37°C. Meanwhile, the elimination half-life and mean residence time were significantly increased during hypothermia (184±21 vs 81±8 minutes, and 3.6±0.8 vs 1.5±0.17 hours, respectively).

Collectively, these results demonstrated that morphine metabolism is inhibited during hypothermia, and careful monitoring the drug concentration and response is necessary. The reduced activity of UGT during hypothermia is a likely mechanism underlying the inhibition of metabolism. However, study on alternative UGT substrates, morphine pharmacodynamics during hypothermia will be necessary for further confirmation.

#### **1.3.4 Transporter function in therapeutic hypothermia**

A growing body of evidence has demonstrated a significant role of various transporters as effectors of both drug distribution and excretion. The major drug transporters, the ATP binding cassette (ABC) family are the major family involved in drug distribution, which include transporter ABCB1 or MDR1 P-glycoprotein (PGP). PGP has considerable influence on the systemic absorption and pharmacokinetics of drugs, especially for many calcium blockers and antiarrhythmic drugs used in critical care medicine. A previous study by Jin et al. demonstrated that MDR1-mediated transport of digoxin was decreased in vitro at temperatures of 32°C (Jin et al., 2006). The transporter net ratio from basal side to apical side direction for digoxin decreased approximately 50% in lower temperature (7.5 vs 14), suggesting that MDR1 transport activity was reduced at 32°C. No significant difference in passive diffusion process was observed at 32°C in this study. In addition, the transport of inulin, as a marker of paracellular pathway, showed no effect of temperature at 25°C, suggesting that the tight junction was not affected during therapeutic hypothermia. Therefore, this study indicated that therapeutic hypothermia cause a suppression of active MDR1 transporter, whereas, no alterations in passive diffusion occurs at 32°C.

Previously discussed liver blood flow marker ICG is also a transporter substrate. ICG is excreted into bile through multiple different transporters including organic anion transporter (OATP), and multidrug resistance associated protein (MRP2). Biliary clearance of ICG decreased significantly to 46% in hypothermia at 32°C ( $1.94 \pm 0.26$  vs  $4.18 \pm 0.63$  ml/min) (Daemen et al., 1986).

Collectively, these studies suggest that the activity of PGP reduces during hypothermia, while passive diffusion is not altered by cooling. ABC transporters require energy in the form of adenosine triphosphate (ATP) to translocate substrates across cell membranes. Thereby, these results suggest that there is a reduction in energy dependent processes during hypothermia. More studies are needed for determining the effect of hypothermia on transporters MDR1, OATP and others.

### **1.3.5 Renal elimination in therapeutic hypothermia**

Renal elimination function can be divided into filtration, active secretion and re-absorption. The balance between renal clearance and metabolism is determined by physical-chemical properties of the drug (Smith et al., 1996). Gentamicin is a commonly used IV antibiotic that is eliminated largely by passive filtration with a minor contribution by tubular secretion. A previous study by Liu et al. evaluated gentamicin in infants with HIE (Liu et al., 2009). This study showed that the mean trough serum gentamicin concentration of hypothermia and normothermia groups were similar ( $2.19 \pm 1.7$  vs  $2.30 \pm 2.0$  mg/L). The clearance of gentamicin was not altered by hypothermia at 33.5°C. As expected, a significant correlation was found between high serum gentamicin concentration and impaired renal function. Plasma creatinine concentrations were closely correlated ( $r^2=0.36$ ) with trough serum concentration in both hypothermia and

normothermia groups. In an animal study, gentamicin pharmacokinetics was studied during hypothermia at 35°C in hypoxia piglets. Pharmacokinetics was evaluated after three gentamicin doses: before hypoxia-ischemia, after hypoxia-ischemia during mild hypothermia or normothermia, and during normothermia 48 h after the first dose. The study found that hypoxia-ischemia altered the renal function and the gentamicin clearance correlated with the creatinine plasma concentration ( $r=0.89$ ) as well as kidney pathology score ( $r=0.55$ ). There was no significant change in gentamicin pharmacokinetic parameters at 35°C compared to 39°C. Therefore, mild hypothermia after hypoxia-ischemia does not affect gentamicin pharmacokinetics over clinically employed therapeutic hypothermia temperatures (Satas et al., 2000). Although studies which evaluated temperatures below 30°C have shown reduced gentamicin clearance due to a reduced cardiac output and decreased GFR (Koren et al., 1985), these reductions are not expected over the clinically relevant 32-35°C temperature range.

Pancuronium is another drug that is primarily excreted through kidney filtration. Sixty to eighty percent of pancuronium is excreted through the kidneys and the remainder is metabolized in the liver and excreted in the bile. In a cat model, pancuronium was studied by Miller et al. in cat at 39°C, 34°C and 29°C (Miller et al., 1978). Volume of distribution of central compartment and total volume of distribution of pancuronium at steady state were both decreased at 34°C compared to 39°C. Total plasma clearance was found to be significantly reduced at 29°C compared to 39°C, while there was no difference of pancuronium clearance at 34°C vs 39°C ( $10.7\pm 0.9$  vs  $10.9\pm 1.5$  ml/kg/min) observed. There was a markedly reduced urinary and biliary excretion of pancuronium only when temperature was below 30°C. Fluorescein isothiocyanate (FITC)-dextran (FD-4), an index of GFR has been evaluated in the rat model during therapeutic

hypothermia at 32°C (Nishida et al., 2007). FITC-dextran is eliminated by passive diffusion. Plasma concentrations were almost equal at all time points between 37°C and 32°C. The total clearance and renal clearance at 32°C and 37°C were also comparable ( 2.10±0.14 vs 1.88±0.13 ml/min for total, and 1.57±0.09 vs 1.45±0.05 ml/min for renal), while both were significantly decreased only at a temperature of 28°C (Nishida et al., 2007). Collectively, these studies suggested that the kidney is able to maintain GFR during mild and moderate therapeutic hypothermia (30-35°C) in spite of possible variations in the systemic circulation. Therefore, renal passive filtration is not likely to be altered by mild hypothermia.

With respect to renal tubular secretion, PSP (phenolsulfonphthalein), a hydrophilic dye, has been used as a tool to assess renal function in preclinical studies. PSP is excreted into the bile and urine as a free form or conjugative metabolite. A previous study by Nishida et al. has shown that the plasma concentrations of PSP were increased significantly in the hypothermic groups (at both 32°C and 28°C) compared to the normothermic group at 37°C (Nishida et al., 2007). The plasma AUC of PSP in hypothermic group at 32°C was significantly larger than those in the normothermic group (1487.9±227 vs 788.6±0.9 µg·min/mL). The total clearance of PSP decreased about 42% at temperature 32°C (0.87±0.1 vs 1.51±0.15ml/min). In addition, both plasma MRT and bile MRT of PSP in the hypothermic group were significantly longer than those in normothermic group (95.1±15.7 vs 49.9±4.7 minutes for plasma MRT and 88.2±12.1 vs 49.5±0.9 minutes for bile MRT). Furthermore, biliary and urinary recovery rates of PSP metabolite excretion were calculated as 37°C (66%, 34%) vs 32°C (75%, 25%), showing a tendency of decrease in urinary contribution by low body temperature. Therefore, PSP clearances (bile, urine and metabolites) in the hypothermic group were decreased, suggesting that

hypothermia reduces active tubular secretion of PSP (Nishida et al., 2007). In addition, this study also suggested that active renal transporter would be attenuated at 32°C due to a failure in energy dependent drug secretion.

In summary, these studies suggest that active, energy requiring processes such as tubular secretion are reduced during therapeutic hypothermia, whereas passive filtration is not significantly altered in mild and moderate hypothermia at 30°C-35°C. The effect of hypothermia on hepatic metabolic pathway, phase II enzyme, transporter and renal elimination based on the current evidence has been summarized in Figure 1-2.

### **1.3.6 Current evidence on drug response changes during therapeutic hypothermia**

Besides the effect of hypothermia on drug metabolism and pharmacokinetics, drug response changes during hypothermia have been reported in both clinical and preclinical studies (Table 1-4).

The drug responses to neuromuscular blockers including vecuronim, atracurium, neostigmine, rocuronium and tubocurarine have been evaluated during hypothermia. Previous study by Heier et al. has demonstrated that mild hypothermia increased the duration of action and time for spontaneous recovery from vecuronium-induced neuromuscular blockade in patients undergoing elective surgery (Heier et al., 1991). Decreasing core temperature correlated with an increasing duration of action of the second infusion of vecuronium ( $R^2=0.58$ ,  $p<0.005$ ). There was no difference between hypothermic and normothermic subjects as measured by the steady state concentration producing 50% depression of twitch tension on the train-of-four test. Thus this

study has suggested that hypothermia mediated increases in the duration of action of vecuronium was not due to the pharmacodynamics response, but more likely due to a change in pharmacokinetics of vecuronium. Similarly, a previous study by Caldwell et al. has also demonstrated the increased duration of action of vecuronium during hypothermia (Caldwell et al., 2000). Hypothermia decreased the rate constant for vecuronium equilibration between plasma and effect site (0.023 per °C/min), and increased the slope of the concentration-response relationship by 0.43 per C indicating that recovery of neuromuscular function has been delayed by hypothermia. This study suggested that reduced metabolism and rate of effect site equilibration both contributed to the prolonged response. Previous study by Leslie et al. has shown that mild hypothermia at 34°C increased the duration of atracurium significantly (Leslie et al., 1995). Core hypothermia prolonged the time to recovery of the first twitch in the train-of-four to 10% of its control value (TI = 10%) after atracurium administration by -60% (p< 0.05), from 44± (SE 4) min to 68 ± (SE 7) min. Beaufort et al. has shown that hypothermia at 30.4 ±0.8° C prolonged the duration of action of rocuronium and delayed spontaneous recovery. The altered pharmacokinetics, such as a decreased clearance was a primary contributor (Beaufort et al., 1995). Ham et al. reported a prolonged duration of action d-tubocurarine at body temperature of 35.8°C and 31.9°C in neurological patients (Ham et al., 1978). Meanwhile, the efficacy of neostigmine as an antagonist of vecuronium induced neuromuscular block was not altered by mild hypothermia at 34°C and clearance of neostigmine was not changed (Heier et al., 2002). When temperature was reduced below 30°C, pancuronium neuromuscular block was prolonged because of an increased sensitivity of the neuromuscular junction to pancuronium and delayed biliary and urinary excretion (Miller et al., 1978). A 2°C reduction in body temperature may double the duration of neuromuscular blockage. Collectively, these studies suggested that

reduction in body temperature to mild and moderate hypothermia may prolong the duration of neuromuscular blockers. It is important for clinical practitioners to be aware of this prolonged response and to increase pharmacodynamics monitoring of these patients in both the cooling and rewarming phases of therapeutic hypothermia. In addition, the observed prolonged response of neuromuscular blockers during hypothermia is more likely due to the reduced metabolism and increased drug concentrations during hypothermia. Interested reader can further refer to Heier et al. review paper in 2006 which has focused on the response to neuromuscular blocking drugs (Heier et al., 2006).

Besides neuromuscular blockers, the responses for anesthetics isoflurane and morphine have been evaluated during hypothermia. Liu et al. observed a reduction in isoflurane dosing requirements in children by 5.1% per °C. Isoflurane minimum alveolar concentration (MAC) values were  $1.69 \pm 0.14\%$ ,  $1.47 \pm 0.10\%$ , and  $1.22 \pm 0.15\%$  at 37° C, 34° C, and 31° C, respectively (Liu et al., 2001). Isoflurane pharmacokinetics was not evaluated in this study. Therefore the pharmacokinetic change in clearance and volume of distribution as it relates to the effect is unknown. In addition, isoflurane is metabolized by CYP2E1 enzyme, and a decrease in metabolism by CYP2E1 may underlie the prolonged drug response and reduced dosage requirement. However, additional studies are needed to determine the correlation between CYP2E1 activity and isoflurane response.

Previous study by Puig et al. has shown that the potency of morphine was significantly decreased at 30°C and increased at 40°C when compared with its potency at 37°C in guinea pig ileum (Puig et al., 1987). This study demonstrated that there was a large decrease in the affinity of morphine

for its receptor as was evidenced by a six-fold increase in the dissociation constant at 30°C. This study suggested that the affinity of morphine for the  $\mu$ -receptor decreased as temperature 30°C. In another animal study by Bansinath et al., morphine response during hypothermia has been evaluated in dog model. After a single intravenous bolus injection of 1 mg/kg, morphine resulted in a significant and sustained decrease in mean arterial pressure in hypothermia, but not in normothermia. The hypotension was observed throughout the experiment in 30°C group but not in the normothermic group. During hypothermia, 10 minutes after morphine administration, the MAP was 35±5 mm Hg vs 114±5 mm Hg in normothermia. The decrease in MAP observed in 30°C was statistically different from 37°C until 3.5 hours. The correlation coefficient between plasma morphine levels and the mean percentage decrease in MAP was 0.98 for the hypothermic group. Similarly, the correlation coefficient for CSF morphine levels and percent decrease in MAP was 0.95 in the same experimental group. Therefore, this drug response change was likely possibly due to the reduced morphine metabolism. Collectively, reduced body temperature in therapeutic hypothermia range prolonged the anesthetic drug response. Hypotension has been observed as one of the most common side effects for hypothermic patients. Prolonged ICU stay and prolonged respiratory depression may mask and alter the benefit effect from hypothermia. Clinical drug monitoring for side effects of series of anesthetics is warranted until a full understanding of drug PK and response during TH is determined.

$\beta$ -Adrenoceptor response during hypothermia was studied by Han et al. The study has shown that the physiological cardiovascular responses mediated by the  $\beta$ -adrenoceptor were significantly diminished during core hypothermia at 33°C (Han et al., 2008). In this study, the dose dependent positive inotropic and chronotropic effects of isoproterenol observed at 37°C were diminished as

core temperature lowered to 33°C or below. The lack of ability to increase cardiac output in hypothermia may also partially due to the increase in total peripheral resistance at temperature below 33°C. The changes of binding affinity or downstream intracellular signaling related to cAMP (cyclic adenosine monophosphate) alteration have been suggested as the underlying mechanism of this effect (Han et al., 2008). However, previous study by Williams et al. has shown that the positive inotropic responses to orciprenaline of paced left atria and papillary muscles were actually potentiated at the lower temperature at 30°C (Williams et al., 1982). A similar hypothermia-induced supersensitivity was observed for the positive chronotropic response of right atria. While the  $\beta$ -adrenoceptor has two types  $\beta$ 1 and  $\beta$ 2, these studies suggest that hypothermia-induced supersensitivity occurs only at the  $\beta$ 1-adrenoceptors not  $\beta$ 2, indicating a fundamental temperature-dependent difference between the two receptor types.

In summary, the changes of drug response for neuromuscular blockers, volatile anesthetics, opiates, and  $\beta$ -adrenoceptor blockers have been observed during hypothermia. Reduced metabolism and clearance may explain part of the response changes. The reduced affinities to  $\mu$ -receptor and changes in downstream signaling to the specific effect on  $\beta$ 1-adrenoceptors have been reported. Careful clinical pharmacotherapeutic monitoring during hypothermia treatment is necessary to prevent the potential therapy-drug interaction and toxicity caused by the changes in both drug concentration and in drug response during hypothermia.

### **1.3.7 Rewarming**

The third step of therapeutic hypothermia is passive rewarming. During the rewarming phase, physiological functions are restored in homeothermic organism after they have been suppressed

by cold. The effect of hypothermia on drug metabolism is also reversible after rewarming. A theoretical time course model of CYP activity during hypothermia and after rewarming was suggested by Tortorici et al. (Tortorici et al., 2007). The aforementioned phenytoin study demonstrated that the clearance in the rewarming phase was significantly increased from hypothermic values (Iida et al., 2001). Similarly, phenobarbitone, the metabolite rates of excretion (hydroxyphenobarbitone, conjugated hydroxyphenobarbitone and phenobarbitone-N-glycoside) increased substantially (increase 141, 63 and 114%, respectively), during rewarming (Kadar et al., 1982). It is recommended that the body may be rewarmed slowly at a temperature of 0.3 - 0.5°C (1 -- 2°F) every hour to a target temperature of 36°C (Tran et al., 2010; Oddo et al., 2006; Arpino et al., 2008). It may take more than 8 h for passive rewarming. The plasma concentration of fentanyl at 6 h after rewarming remained increased compared with baseline ( $6.3 \pm 0.6$  vs  $< 6.0$  ng/ml,  $p < 0.05$ ) (Fritz et al., 2005). This suggests that the alterations of drug metabolism may exist during rewarming periods. Currently there is lack of studies that focused on the rewarming phase. Instead, many clinical study results combined both effects during hypothermia and after rewarming. For long half-life drugs, the rewarming phase might be even more important due to prolonged elevations in drug concentration even after restoration of drug receptor responsiveness.

### **1.3.8 Conclusion**

Our current understanding suggests that hypothermia has multiple beneficial effects after global ischemic injury. Besides the benefit of hypothermia, the possible side effects associated with hypothermia are unavoidable. The effects of hypothermia on drug metabolism and disposition may increase the probability for unanticipated toxicity, which could limit the putative benefit of

this novel therapy. Recent evidence suggests that the specific alterations of drug metabolism and the magnitude of the change observed during hypothermia can be metabolism and elimination route specific. The effects of hypothermia on drug response including muscle relaxant, volatile anesthetics, opiates and  $\beta$ -adrenoceptor agonist have been reported. Pharmacokinetic and pharmacodynamic alterations during hypothermia may both contribute to the resultant effect. Available evidence demonstrates that mild hypothermia decreases the clearance of a variety of drugs with apparently little change in drug protein binding. Future studies on specific CYP probe's metabolism and receptor response are needed. By defining the impact of mild hypothermia on drug metabolism, disposition, and response in experimental models as well as thorough clinical research is needed. In doing so, drug dosing during therapeutic hypothermia can be optimized to facilitate the maximal benefits and minimize side effects of this therapeutic intervention.

Table 1-3: Drug concentration and pharmacokinetic alterations in therapeutic hypothermia with their specific metabolism and elimination pathway

Metabolic pathway	Temperature/subject population	Investigated Drug	concentration/PK parameters estimates
Flow/CYP2B/UGT	34°C/healthy volunteers	Propofol	Css↑ 28%, Inter-compartment CL↓ with temperature
Flow/CYP3A	31.6°C/piglets	Fentanyl	Plasma conc↑25±11%, temperature and CYP3A4 dependency
Flow/bile/OATP	32.5°C/ male Wistar rats	ICG	CL↓80%, MRT↑ 2-fold
Flow/bile/OATP	32°C/ rats	ICG	Total CL↓ to 47%, AUC↑2-fold, CLb↓
CYP3A	Moderate 32-34°C/TBI patients	Midazolam	Plasma concentration ↑, Vd↑83%, CL↓, Ke↓
CYP3A	35.5-36.5°C /healthy volunteers	Midazolam	CL↓ 11% per degree
CYP3A/PGP	<35, 35-35.9,36-36.9°C/volunteers	Vecuronium	CL↓11.3% per degree
CYP3A	32°C /In vitro microsome	Ethylmorphine	Temperature dependent reaction, CYP3A4 activity ↓ to 69%
CYP2C9/CYP2C19	34 °C/ brain damage patients	Phenytoin	AUC↑180%, MRT↑180%, CL↓67% and Ke↓50%
CYP2C9/3A	34.5°C/ healthy volunteers	Neostigmine	V1↓38%, no change in CL
CYP2C9/Renal	32.5°C/ male Wistar rats	S-acenocoumarol	CL↓26%
CYP2C19/UGT	30-31°C /TBI children	Phenobarbital	Ke↓, renal metabolite excretion↓, Vd↑25%
CYP2D6/2B	Server <32°C/9 out of 11 children	Pentobarbital	CL↓, Vd↓20%, no change in T1/2
CYP2D6/2B	30°C/perfused rat liver	Pentobarbital-2-c14	CLh↓50%, CLb↓ 71%
CYP2D6/Renal	30.4°C/ neurosurgical patients	Rocuronium	CL↓ to 51%, MRT↑1.9 fold
CYP2E1	30°C/cardiac arrest rats	Chlorzoxazone	Km↑116%, Clint↓44%,CL↓ 54%, Ke↓66%
UGT	33-34°C/ HIE infants	Morphine	CL↓, potential toxic concentration in hypothermia
UGT	30°C/dog	Morphine	Conc in plasma and CSF↑, CL↓ 70%,T1/2β↑2-fold, MRT↑, Vd↓
Transporter PGP	32°C /in vitro kidney epithelial cell	Digoxin	Direction from B to A ↓50%
Renal filtration	33.5°C/HIE infants	Gentamicin	No change in CL, high concentration related to renal impairment
Renal filtration	35°C/hypoxia newborn pig	Gentamicin	No change in CL
Renal filtration/CYPs	32°C/cats	Pancuronium	No change in CL, Vss↓
Renal filtration/GFR	32°C/ rats	FD-4	No change in pharmacokinetics
Renal tubular secretion	32°C/ rats	PSP	Total CL↓ 42%, plasma AUC↑2-fold, MRT↑, renal secretion ↓

Table 1-4: Current evidence on drug response change in therapeutic hypothermia

Pharmacological effect	Temperature/subject population	Investigated Drug	Drug response/PD estimates
Muscle relaxant	34.5°C/patients undergoing surgery	Vecuronium	Recovery time↑, duration of action↑
Muscle relaxant	<35, 35-35.9,36-36.9°C/volunteers	Vecuronium	Response↑, equilibration rate↓
Muscle relaxant	34°C/healthy volunteers	Atracurium	Response↑, duration of action↑
Muscle relaxant	34°C/healthy volunteers	Neostigmine	No change in efficacy and duration
Muscle relaxant	30.4°C/ neurosurgical patients	Rocuronium	Duration of action↑
Muscle relaxant	31.9°C/neurological patients	D-tubocurarine	Duration of action↑
Volatile anesthetic	34, 31°C/children	Isoflurane	Dose requirement↓ 5.1% per °C. MAC↓
Opiate anesthetic	30°C /dog	Morphine	Hypotension incidence↑
Opiate anesthetic	30°C/ guinea pig ileum	Morphine	IC50↓, the affinity to μ-receptor↓
β-adrenoceptor agonist	33°C/rats	Isoproterenol	HR↓, CO↓, SV↓, LV dP/dtmax↓
β2-adrenoceptor agonist	30°C/ guinea-pig	Orciprenaline	β1 adrenoceptor sensitivity↑

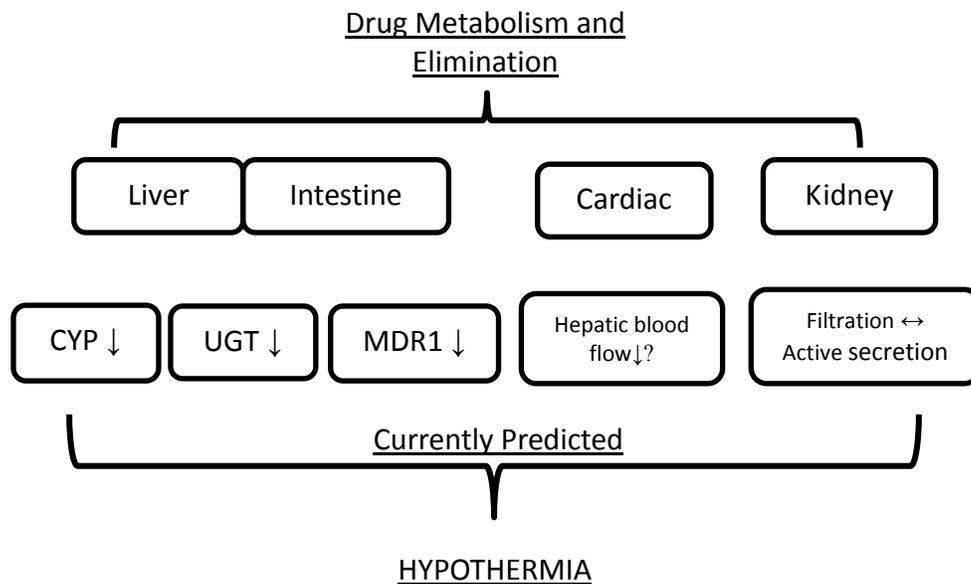


Figure 1-2: The effect of hypothermia on drug metabolism and elimination pathway

## 1.4 SIGNIFICANCE AND SPECIFIC OBJECTIVES OF THE THESIS RESEARCH

### 1.4.1 Significance

Mild therapeutic hypothermia is emerging clinically as a neuroprotection therapy for cardiac arrest victims and other critical illness. However the effects of therapeutic hypothermia in combination with the critical illness on drug disposition and response have not been fully elucidated. The effects of hypothermia on drug metabolism and disposition may increase the probability for unanticipated toxicity, which could limit the putative benefit of this novel therapy. Previous studies have found significant elevated drug levels and prolonged drug response during hypothermia, for drugs such as phenytoin, neostigmine, vecuronium, morphine, propofol, and fentanyl. With new interventions comes an increase in the care of optimizing drug therapy through rationale concentration based dosing adjustments in order to prevent side effects in these highly vulnerable patients. By defining the impact of mild hypothermia on drug metabolism, disposition and enzyme regulation in experimental models as well as through clinical translational research, drug dosing during therapeutic hypothermia can be optimized to facilitate the maximal benefits and minimize side effects of this therapeutic intervention.

In addition, during hypothermia post CA, the alterations in normal drug metabolism, can be quite complex due to suppressed effects from heart failure, multiple organ dysfunction and temperature. Various pathophysiological disturbances including hepatic ischemia and changes in hepatic blood flow may significantly alter the PK behavior of drugs. Therefore a thorough understanding of both the temperature and CA pathophysiologic interaction on individual CYP isoform activity is necessary to delineate the alterations in drug metabolism and disposition.

Surprisingly, the specific/quantitative changes of drug metabolism and disposition during hypothermia after CA are largely unknown. Therefore, to investigate the possible alteration on drug metabolism caused by CA injury as well as CA-temperature interaction is necessary.

Currently population based pharmacokinetic modeling (nonlinear mixed effect modeling) has been recognized useful in critical care pharmacology, anesthesia and pediatric pharmacology. Population PK modeling describes the complex relationship between multiple factors (such as diseases, treatment, temperature, drug combination covariates) and drug metabolism and disposition from various clinical and animal studies. In order to gain new insight into optimizing pharmacotherapy in these critical ill patients, it is the also a goal of this thesis research to develop population PK models that serves as a mathematic representation of physiological and pharmacological phenomena and provide the estimation and prediction of the hypothermia effect as well as disease interaction on drug metabolism and distribution.

#### **1.4.2 Specific objectives of this research**

The current understanding of the possible TH effect on drug concentrations led to the need for evaluation of multiple CYP isoforms in one set of studies with a disease model. Therefore, the first objective of the study is to investigate hypothermia CYP isoform specific effect (CYP2C, CYP2D, CYP3A and CYP2E1) and disease interaction by studying multiple hepatic metabolic enzymes and the PK of their substrates during hypothermia in experimental cardiac arrest rat model. This objective is addressed in Chapters 2 and 3. The study utilized advanced analytical assays, in vivo probe drug metabolism analysis, asphyxia CA model and population modeling analysis.

Within all CYP isoforms, CYP3A4 is the important isoform, therefore, the second objective of this study is to investigate the effect of mild hypothermia on the single isoform CYP3A activity in healthy human volunteers. This objective has been completed in Chapter 4. This study utilized the well-known probe drug midazolam metabolism profile, approach to induce mild hypothermia in healthy volunteers and population modeling and simulation for a mathematic prediction of the correlation between the changes of temperature and midazolam clearance.

Realizing the difficulty of conducting a further PK study with critical ill patients, the third objective of this study investigated the feasibility and accuracy of using microdosed probe cocktail approach in evaluating the phenotyping of multiple CYP enzymes (CYP1A2, CYP2C19, CYP2D6 and CYP3A4) and renal secretion in healthy human volunteers. This objective has been described in Chapter 5. This study utilized advanced analytical assays, PK simulation, clinical pharmacology study design, as well as a summary of literature reported PK parameters for each probe drug used in higher dose. The result will be greatly useful for further clinical study design determining the drug metabolism and disposition change in critical ill patients.

Finally, as an extension of our previous research with CYP probe drugs, we sought to evaluate the effect of therapeutic hypothermia on the disposition of clinically relevant drugs in susceptible patient populations. Phenytoin is widely used clinically, has a narrow therapeutic index, and follows non-linear pharmacokinetics. Therefore, the fourth objective of this study is to determine the effect of therapeutic hypothermia on population pharmacokinetics of phenytoin in pediatric traumatic brain injury patients. This objective has been described in Chapter 6. This project was focused on determining the hypothermia effect on phenytoin nonlinear elimination, the

estimation of the temperature correlation with  $K_m$  and  $V_{max}$  by population modeling and prediction simulation. The result of the study is greatly helpful in providing rational for possible dosage adjustment guidelines for phenytoin in clinics.

Overall, this thesis research provided an essential proof of concept to support subsequent clinical studies aimed at optimizing the specific dosage guidelines in the critically ill patient population receiving therapeutic hypothermia based on specific drug metabolic pathways, the patient disease states and specific temperatures and durations of hypothermia (Figure 1-3). It also provided essential translational tools and methodology tools for others who interested in clinical and preclinical PK study design and data analysis.



Figure 1-3: Rational for future pharmacotherapy for critical ill patients receiving hypothermia

## 2.0 MULTIPLE PROBE DRUGS BIOANALYTICAL ASSAY BY UPLC/MS/MS-FOUR DRUG COCKTAIL

### 2.1 INTRODUCTION

Analytical methods play a vital role in supporting every facet of the drug research, discovery and development and approval process. For this thesis research, in order to assess CYP isoform specific effect of therapeutic hypothermia, a cocktail was designed that contains multiple probe drugs together and bioanalytical assays were established for measuring multiple probe drug concentrations simultaneously. The application and feasibility of cocktail cytochrome P450 (CYP450) probes was reported first by Breimer and Schellens around twenty years ago (Breimer et al., 1990). Since then many interests has been drawn to the CYP probes cocktail applications for in vitro drug screening and clinical assessment for CYP activity, drug-drug interaction, genotyping in different patients populations, new chemical screening, drug-therapy interaction, and disease metabolism interaction (Breimer and Schellens 1990; Tucker et al., 2001). The advantages of cocktail approach include less inter-subject and inter-occasion variability, and relatively less amount of work and costs compared to analyzing single drug separately. Despite the advantages, some of the drawbacks of the cocktail approach may include potential drug-drug interactions among probes, demand for a higher sensitivity analytical equipment, the discrepancy between in vitro and in vivo due to the factors such as absorption, protein binding, transporter effect and lack of preclinical pharmacokinetics data for the evidence of safety and validation of cocktail combination (Paolini, et al., 1993; Zhou et al., 2004; Davit et al., 1999 ).

In this thesis study, we have established two set of cocktail assays and they both involved in new probe drugs combination. In this chapter, I am discussing the establishment of the first bioanalytical assay, which will be used later used in a preclinical animal study. Compared to previous reported cocktail studies, we have made three additional advances as: 1) a strategy of low dosages of all probes has been applied, since lower dose can potentially minimize the drug-drug interaction, toxicity and improve the probe specificity for the metabolic pathway; 2) a sensitive, rapid and reliable analytical method by advanced ultraperformance liquid chromatography-triple quadrupole mass spectrometry (UPLC/MS/MS) has been used for simultaneously measuring all probe drugs and metabolites in one run; and 3) an intravenous (IV) approach is used for all probe drugs rather than given orally to avoid the possible absorption, possible extra-hepatic metabolism, and active transport effect from oral route.

The purpose of this assay establishment is to be able to measure the plasma concentration of probe substrates for four different CYP isoforms simultaneously and be able to evaluate these specific CYP isoforms activities from one set of experiment. Since no previous method has been set up in the lab or in literature, this study has first reported of this assay development. Bio-analytical assays should be selective and sensitive and it involves multiple steps to get to the assay validation. I summarized the method development and validation into multiple steps, which has been shown in Figure 2-1.

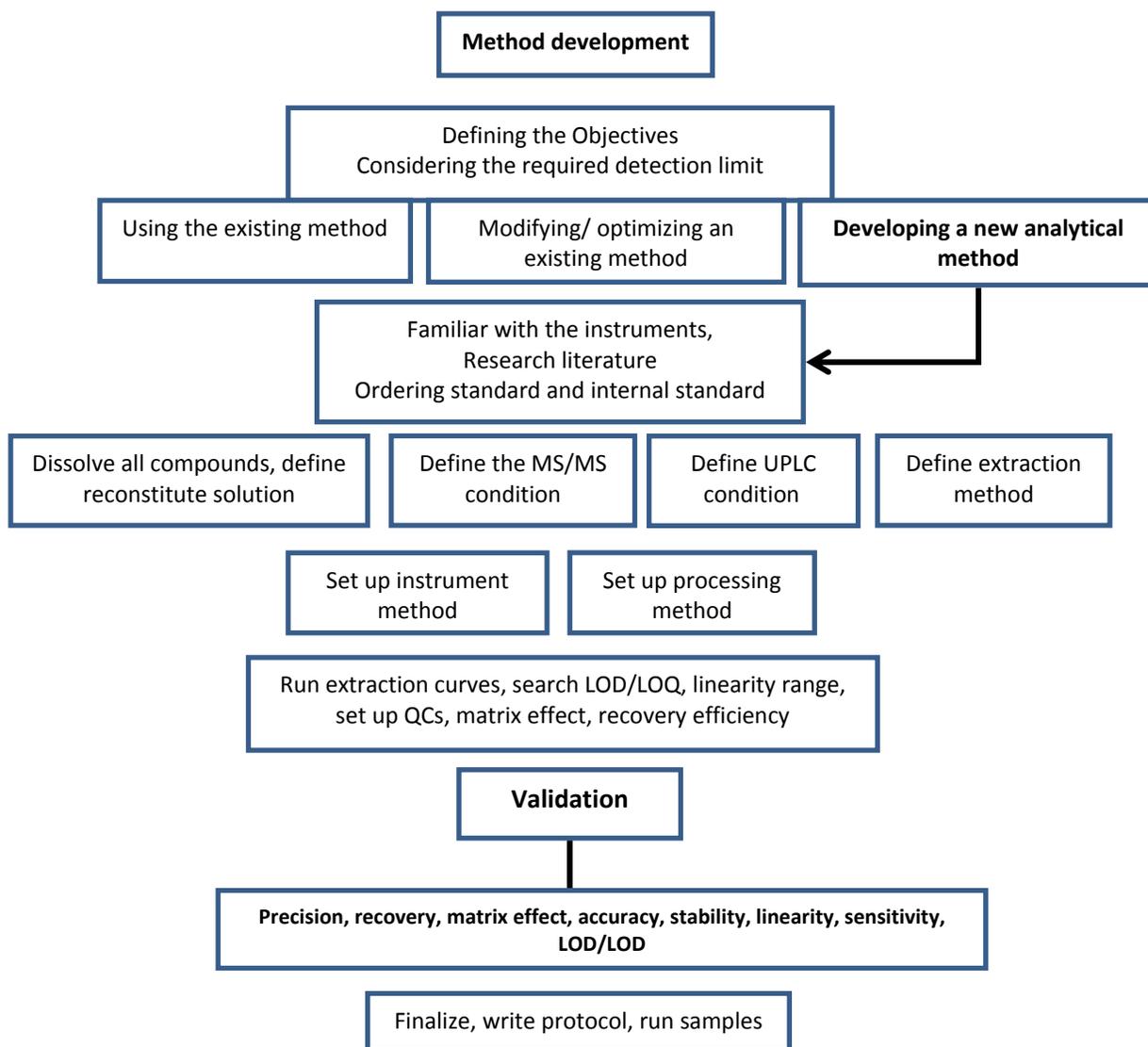


Figure 2-1: Method development and method validation multi-steps

## 2.2 EXPERIMENT AND METHOD VALIDATION

### 2.2.1 Chemicals and reagents

Dextromethorphan (DEX), dextrorphan (DOR), chlorzoxazone (CHL), 6-hydroxychlorzoxazone (6OHCHL), diclofenac (DIC), 4-hydroxydiclofenac (4OHDIC),  $\beta$ -glucuronidase (from *Helix Pomatia*) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 1-Hydroxymidazolam (1OHMDZ) and midazolam-d4 standards were purchased from Cerilliant Corp (Round rock, TX, USA). Dextrorphan-d3, chlorzoxazone-d3, and 4-hydroxydiclofenac-d4 were purchased from Medical Isotopes, Inc (Pelham, NH, USA). Midazolam (MDZ, 5mg/mL) injection (Versed, Roche, USA) was used. Blank rat plasma (pooled, heparized) was purchased from Innovative Research, Inc (Novi, MI, USA). High purity solvent acetonitrile and water were purchased from Honeywell B&J brand (Muskegon, MI, USA). Formic acid (~98%) was bought from Fluka (Germany). Oasis 1cc HLB (hydrophilic-lipophilic balance) solid phase extraction cartridges were purchased from Waters (Milford, MA, USA).

### 2.2.2 Instrumentation and conditions

Waters UPLC systems with ACQUITY BEH C18 1.7 $\mu$ m $\times$ 2.1mm $\times$ 50mm column plus UPLC guard column (1.7 $\mu$ m $\times$ 2.1mm $\times$ 5mm) were used to separate the analytes. Column temperature was maintained at 50°C. The mobile phase consists of 0.1% formic acid in water (A) and pure acetonitrile (B), gradient from 90:10 (A:B) to 70:30 (A:B) within 3 minutes and come back to 90:10 at 6.0mins. Flow rate was used at 0.25mL/min. Strong needle wash: pure acetonitrile, weak needle wash: 10% acetonitrile in water. All injection volumes were 7.5  $\mu$ L. (1.0ng/mL= 7.5 pg/column)

Thermo triple-quadrupole mass spectrometer, equipped with electrospray ionization (ESI) source was used for mass analysis and detection (Thermo Fisher Scientific, San Jose, CA). ESI operated at six positive (spray voltage 4.0kV) and six negative ionization (spray voltage 4.0kV) modes, with argon as the collision gas at 1.2mTorr. Other working parameters set as follows: capillary temperature 300°C, sheath gas pressure 60 psi, auxiliary gas pressure 55psi. The mass peak widths were 0.7 Da for both Q1 and Q3. MS/MS condition and tuning were performed by a T-connection with analyte and mobile phase (running at 0.25mL/min) entering the cone together. The intensities of fragment ion was monitored and optimized to the maximum by using the Quantum Tune master software. Selected reaction monitoring was used for quantification using the product ion with the highest sensitivity. Scan rate was set at 0.01s/scan. Xcalibur version 2.0.6 software was used for data acquisition and analysis (ThermoFinnigan, San Jose, CA) (Figure 2-2).

### **2.2.3 UPLC/MS/MS assay development**

#### ***2.2.3.1 Calibration standard and quality control sample***

All standard stock solution of probes and metabolites and internal standards are 1.0mg/ml or 100ug/ml (for midazolam-D4) in methanol and stored at -20°C for 2 months. All glassware used for preparation of calibrations and samples were silanized tubes. The four probe parents drug were combine together in one cocktail stock with concentrations at midazolam 1.0µg/ml, dextromethorphan 2.5µg/ml, chlorzoxazone 2.5µg/ml and diclofenac 2.5µg/ml. The four metabolites were combined together in one cocktail metabolite stock with concentrations at 1-hydroxymidazolam 1.0µg/ml, dextororphan 2.5µg/ml, 4-OH-diclofenac 2.5µg/ml and 6-hydroxychlorzoxazone 2.5µg/mL. The four duterated internal standards were combined together

in one cocktail IS stock with concentrations at midazolam-d4 0.1µg/ml, dextrorphan-d3 0.1µg/ml, chlorzoxazone-d3 0.5µg/ml and 4-hydroxydiclofenac-d4 1.0µg/ml. Further dilution of lower concentration of the analytes used 80:20 acetonitrile: water. Quality control (QC) samples were prepared at three different concentrations designated within the low, medium and high concentration ranges of the curve.

### ***2.2.3.2 Sample extraction method***

Solid phase extraction (SPE) is newly developed method for sample concentrate and sample cleaning. Solid phase cartridges simply can be considered as a small short HPLC column, the analyte will be separated from mobile phase and stationary phase. Normally the desired analytes of interest or undesired impurities in the sample are retained on the stationary phase then elute with the appropriate solution which can dissolve or have better affinity to the analytes than the stationary column. The reason for us to choose solid phase extraction is because SPE can increase sensitivity compared to other traditional extraction methods, reduce matrix effects, maximize selectivity, optimize recovery efficiency and provide consistent results. In this study, solid phase extraction method using Waters 1cc Oasis HLB reverse phase cartridge has been used. HLB column provides relatively large range of pKa and working pH range and therefore is suitable for extraction of variety of acids, bases and neutrals compounds.

During the experiment, the cartridges were first activated and equilibrated with 1mL methanol and 1mL of water. After the samples have been loaded, the cartridges were washed with 1mL water and 1mL 5% methanol, discarded the elute. Cartridges were then washed with 2×1mL methanol and the elute was collected. The elute was dried down under constant a gentle stream

of nitrogen at 37°C, the residue was reconstituted with 100µL mobile phase (80:20 acetonitrile: water with 0.1% formic acid) and transferred to a clean autosampler vial.



Figure 2-2: Ultra performance liquid chromatography equipped with triple-quadrupole mass spectrometer (UPLC-MS/MS)

#### **2.2.4 Method validation**

Method validation is a dynamic process intended to make sure as well as satisfy different complementary objectives (especially technical and regulatory). Food and drug administration (FDA), International Conference on Harmonization (ICH) and the US pharmacopeia and National Formulary (USP/NF) consistently define validation of an analytical method as “the process by which a method is tested by the user or developer for reliability, accuracy and preciseness of its intended purpose” (FDA 1994; Winslow et al., 2003). Method validation with respect to the selectivity, linearity, precision, accuracy, limit of detection, limit of quantitation, stability as well as extraction efficiency, the matrix effect, the simultaneous analysis methods for the eight analytes were tested.

Every calibration curve consisted of eight concentrations. Calibration curves were constructed using the analytes/IS peak area ratio vs the analyte concentration. The curves were fitted by linear least squares regression, analysis with weighting factor of  $1/y$ . The sensitivity was described from limit of detection (LOD) and limit of quantitation (LOQ). The LOD is the lowest concentration at which the analytes can be detected. The LOD was determined based on the peak area of lower than 5-digit. The LOQ was determined based on the peak area at this level can be determined with variability less than  $\pm 20\%$  (accuracy of 80-120%) when repeated six times in extracted samples. QC samples at low, medium and high in six replicates were analyzed on the same day to determine the intra-day precision. The process was repeated in three separate days to determine the inter-day precision. Precision was calculated as the coefficient of variation (CV%) of the replicate analysis, whereas accuracy is the percent difference between nominal and observed values (% bias). The acceptable inter-day and intra-day precision and bias were set as  $<20\%$  deviations of nominal concentrations.

The recovery efficiency and evaluation of matrix effect were determined by three different process of QC samples: 1) “neat sample”, which was prepared by spiking the calibration stock directly into reconstitute solution; 2) “post extraction”, which was prepared by adding the matrix (50 $\mu$ L blank plasma) through the extraction cartridges without the analyte, the analytes was then added into the elute, dried down, then reconstituted; 3) normal extraction procedure, prepared QC samples, extracted with solid phase cartridges. The matrix effect was calculated as  $(A_{\text{post-extraction}}/A_{\text{Neat}}-1)\times 100$ . Negative value represents the ion suppression effect. The recovery efficiency of every analyte was calculated as  $A_{\text{extraction}}/A_{\text{post-extraction}}\times 100$ .

QCs in five replicates of each concentration have been prepared to test the stability. The analytes were evaluated for autosampler (72 hr, 4°C), short term (24 hr, room temperature), and long term (2 months, -70°C) stability. The analyte was considered stable when the percent difference between nominal and observed concentrations was within 20% of relative error.

### **2.2.5 Animals treatment and sampling procedures**

Male Sprague-Dawley rats (body weight, 300-350g, 2 months old) were purchased from Hilltop Laboratories (Scottsdale, PA). The animals were maintained on a 12-h light/dark cycle and were allowed free access to food and water. The University of Pittsburgh Animal Care and Use Committee approved the study and animal procedures.

Rats were anesthetized with 0.5-1.0% isoflurane via a nose cone, endotracheally intubated, and mechanically ventilated to maintain PaCO<sub>2</sub> between 35 and 45 mm Hg. Body temperature was maintained with appropriate heating pad (38.0±0.5°C). Rat left femoral artery was cannulated for measurement of mean arterial pressure and arterial blood gases, and the left femoral veins were cannulated bilaterally for drug administration and blood sampling. The cocktail drugs were given as an intravenously bolus of 0.2mg/kg midazolam, 0.5mg/kg dextromethorphan, 0.5mg/kg diclofenac, and 0.5mg/kg chlorzoxazone. The IV formulation was made in saline with ethanol (<20% v/v). The injection volume given to rats was 1.0mL/kg body weight. Saline was injected to flush the line after the drug administration. Blood samples (0.3 mL) were obtained via the femoral venous with a heparinized syringe at baseline, 5, 15, 30, 60, 120, 180, 240, 300, 360 and 480 minutes. Blood samples were immediately centrifuged at 2000g for 7 minutes and the plasma was transferred to a labeled eppendorf tube. Rats were sacrificed via decapitation after 8

hours blood sampling. All samples were kept in freezer during the study and then transferred to -80°C until analysis.

### **2.2.6 Sample analysis**

All plasma samples were thawed on ice, an 20-100µL of rat plasma (20µL for 5 minutes sample, 100 µL for 8 hr sample, 50µL for all others) per sample was added to 900 µL water, a 10µL IS cocktail solution was spiked in, vortex. The samples were extracted using solid phase extraction as previous described. Probe substrates concentrations were calculated based on the validated standard curves and the sample volume difference from final reconstitute volume has been adjusted.

### **2.2.7 Pharmacokinetic estimate and comparison**

Non-compartmental ( $AUC_{0-inf}$ ,  $Cl_s$ ,  $T_{1/2}$ , and  $V_{ss}$ ) pharmacokinetic analysis by WinNonlin (Pharsight, CA) 5.2 was used. Estimated pharmacokinetic parameters ( $Cl_s$ ,  $T_{1/2}$ ,  $V_{ss}$ ) were compared with the previous literature values when these drugs were given in healthy rat at different doses.

### **2.2.8 Data analysis**

All figures (except chromatograms) and data expressed as mean  $\pm$ SD. The figures were generated from Prism 5.0 (GraphPad Software, San Diego, CA.). One-way ANOVA with Bonferroni post hoc was used for all comparison (all pharmacokinetic parameters), a  $p < 0.05$  was considered significant.

## 2.3 RESULTS

### 2.3.1 Probe drugs and dosage selection

In this study, midazolam, diclofenac, dextromethorphan and chlorzoxazone were chosen based on the specificity for the CYP3A, CYP2C, CYP2D and CYP2E1, respectively. In addition, a less possibility of drug-drug interaction, relatively shorter half-life and clinical relevance (Frye et al., 1997; Streetman et al., 2000) are also considered. Previous research showed that chlorzoxazone significantly inhibits the CYP3A-mediated first pass metabolism of midazolam in the gut, in contrast, the intravenous injection of midazolam did not shown any interaction with chlorzoxazone (Palmer et al., 2001; Blakey et al., 2004). Relatively shorter or similar half-lives of four probes helped us design better sampling duration and time points. Dextromethorphan is often used as a CYP2D probe because of its wide safety margin and availability compared with other possible probes such as debrisoquine, and sparteine. In addition, all probe substrates were listed by FDA as preferred probes/reactions for CYP enzymes in vivo and in vitro.

The IV dosage for probe drugs were chosen based on the sensitivity of the developed analysis method and allowing the 8-hr concentration can be detected well above the limit. The dosage used in the study was the lowest dosages compared to previous pharmacokinetic studies in rats. The dosages for each drug was at least five-fold lower than the typical dosage used in rats pharmacokinetics study.

### 2.3.2 UPLC/MS/MS instrument condition

Every analytes was tested both in positive mode and negative mode, then the polarity as well as the collision energy, UPLC conditions related to the highest fragment was chosen. The ion transitions for all 12 compounds, collision energy (20-37ev), polarity (six positive, six negative modes), and retention time (RT) were shown in Table 2-1. Under the experimental condition, the representative chromatograms of a standard extracted calibrate at 0.2ng/mL (for MDZ, 1-OHMDZ, MDZ-d4, DIC, DEX, DOR, DOR-d3, CHL, CHL-d3) and 2.0ng/mL (for 4-OHDIC, 4-OHDIC-d4, 6-OHCHL) were depicted in Figure 2-3.

The total run was 6.0 minutes with no peak interference with others. The retention times for 12 analytes were distributed between 2.64 to 4.16 minutes. The established methods allow a sufficient time to: divert the first 2 minutes into the waste if necessary; to add in more hydrophilic analyte into the cocktail combination; or to push the 12 analytes together for an earlier or later retention time if necessary. In addition, the mass spectrometry scanned positive and negative mode alternatively with 0.01s/scan speed faster than machine default (1.0s/scan), which provided at least 10 points across each peak and therefore better captured the peak shape and improved the sensitivity.

Four duterated compounds midazolam-d4, 4-hydroxydiclofenac-d4, dextrorphan-d3 and chlorzoxazone-d3 have been used for internal standards. In most cases, the duterated compounds ranged from 3-8 mass units heavier than the parent compounds are suitable for using as internal standard. However, diclofenac (MW 294) can be detected at both 294 and 298 mass weight

because of the two natural  $^{35}\text{Cl}$  and  $^{37}\text{Cl}$  isotopes in the structure, therefore diclofenac-d4 (MW: 298) has to be avoided.

### **2.3.3 Method validation**

#### ***2.3.3.1 Linearity and sensitivity***

Calibration curves were prepared in three individual days with the precision and accuracy testing. All probe substrates and metabolite curves have a  $R^2 > 0.99$ . All calibration ranges were listed in Table 1. The LLOQ for eight analytes were between 0.2-5.0ng/mL (1.5-75pg/column). UPLC/MS/MS method significantly decreased the background noise, therefore improved the sensitivity and provided wider range of linearity. Our study and other studies have shown that 500-1000 fold of analysis linearity range are accessible with triple tandem mass spectrometry (Ghassabian et al., 2009, Beaudry et al., 1998).

#### ***2.3.3.2 Precision and accuracy***

The intra-day and inter-day precision were  $< 20\%$  for all QCs. The accuracy (% bias) of all analytes QCs were  $< 13.6\%$ . The good precision and accuracy indicate that the analytical methods are reproducible and reliable (Table 2-2).

#### ***2.3.3.3 Matrix effect and recovery***

The areas of neat samples, post extraction samples and normal extraction samples have been used for calculation of matrix effect and recovery. The matrix effect for every analyte was different and ranged between  $-42.7\%$  to  $+20.2\%$ . There was a matrix ion suppression effect for most analytes (post extraction response was lower than the neat sample response) while there

was matrix improvement effect for midazolam and chloraxazone at medium and high QC levels. For all eight analytes, the recovery (normal extraction/post extraction  $\times 100\%$ ) ranged between 72.5-112.6%. Recovery efficiency indicated that the solid phase extraction method was sufficient to recover  $> 72.5\%$  of compound from standard solution without significant loss in the extraction procedure. In addition, four internal standards, midazolam-d4, 4-hydroxydiclofenac-d4, dextroprhan-d3 and chlorzoxazone-d3 have also been evaluated individually for matrix effect by comparing the area of neat samples and post extraction samples. The results showed the internal standards were well correlated with the characteristics of the parent drugs. These results showed the methods were robust and specific (Table 2-3).

#### **2.3.3.4 Stability**

High, medium and low QC concentrations in five replicates have been utilized to evaluate the stability. Since all calculated relative error was less than  $\pm 15\%$ , no significant loss/gain or interaction in solution of the analytes was observed at any of the investigated conditions. The samples were considered stable within 72 hours at  $4^{\circ}\text{C}$ , 24 hours at room temperature, and 2 months at  $-70^{\circ}\text{C}$  freezer. Therefore, the method can be used to analyze samples under the tested conditions, and considered stable without significant degradation or condensation (Table 2-4).

#### **2.3.4 Time-plasma concentration profiles and pharmacokinetic estimates**

Time-plasma concentration profiles of midazolam, diclofenac, dextromethorphan and chlorazoxazone were shown in Figure 2-4. Pharmacokinetic estimates from non-compartment model were shown in Table 2-5. Multiple previous literature reported  $CL_s$ ,  $T_{1/2}$  and  $V_{ss}$  values for midazolam, diclofenac, dextromethorphan and chlorazoxazone from rats were listed in Table 2-6

for comparison. Compared with other literature preclinical PK parameters for these four drugs, our preclinical rat data from healthy rats are consistent with previous literature reported value.

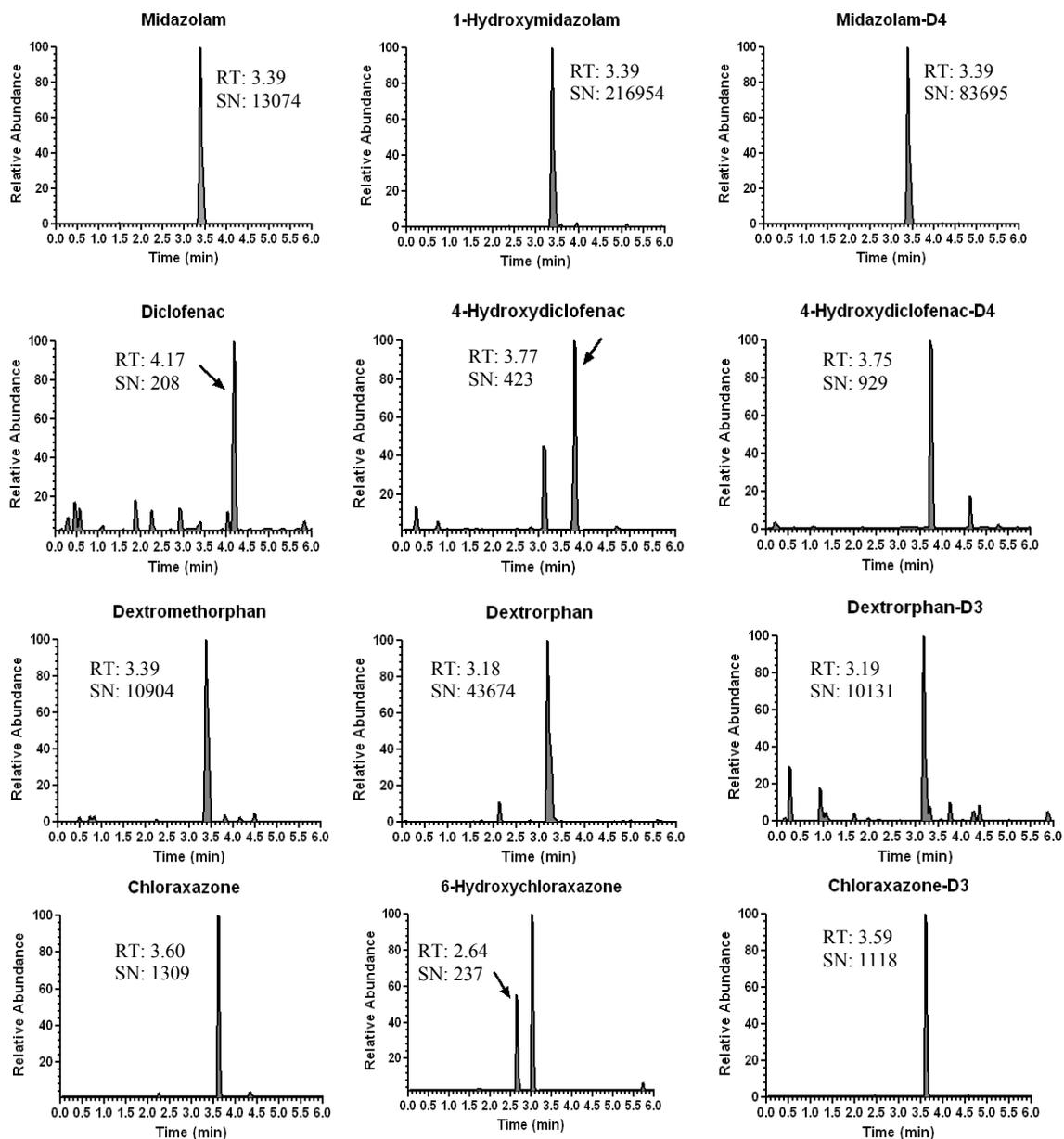


Figure 2-3: UPLC/MS/MS chromatograms of both positive and negative ion analytes resulting from a 7.5 $\mu$ L injection of a low concentration calibration standard (0.2ng/mL for all analytes except for 2.0ng/mL for diclofenac, chloraxazone and 5.0ng/mL for 4-OH-diclofenac, 4-OH-diclofenac-d4 and 6-OH-chloroxazone) at the LOQ in plasma.

Table 2-1: SRM transition method for MS/MS (precursor to product) for four parent drugs, four major metabolites and their respective internal standards

CYP450	Compound	SRM transition	Collision energy (ev)	Polarity	RT (min)
CYP3A1/2	Midazolam	325.7>291	27	+ve	3.39
	1-OH-midazolam	342>324	25	+ve	3.39
	Midazolam-d4	330>295	27	+ve	3.39
CYP2C11	Diclofenac	294>250	25	-ve	4.16
	4-OH-diclofenac	310>230	20	-ve	3.77
	4-OH-diclofenac-d4	314>234	20	-ve	3.77
CYP2D1	Dextromethorphan	272>171	37	+ve	3.39
	Dextorphan	258>157	33	+ve	3.18
	Dextorphan-d3	261>157	30	+ve	3.18
CYP2E1	Chlorzoxazone	168.6>132	22	-ve	3.60
	6-OH-Chlorzoxazone	186>120	25	-ve	2.64
	Chlorzoxazone-d3	171>134	25	-ve	3.60

Table 2-2: Calibration curve ranges, precision and accuracy evaluation of eight analytes (n=6)

	QC	Day1	Day2	Day 3	%bias	Precision (CV%)			LOQ ng/mL	Standard Curves
						Day1	Day2	Day3		
MDZ	5	4.96±0.37	4.88±0.44	4.98±0.17	- 1.2	7.5	9.1	3.4	0.2	0.2-200ng/mL (plasma)
	40	36.2±3.0	39.0±3.9	37.5±3.9	- 6.1	8.3	10.0	10.4		
	200	204±13	212±8.3	198±19.0	2.3	6.4	3.9	9.6		
1-OHMDZ	5	5.12±0.87	5.09±0.36	5.14±0.60	2.3	16.9	7.0	11.7	0.2	0.2-200ng/mL (plasma)
	40	40.4±3.5	41.0±1.9	39.5±4.1	0.75	8.7	4.8	10.4		
	200	197±8.1	198±16.4	204±17.4	- 0.17	4.1	8.3	8.5		
DIC	12.5	11.9±2.4	10.4±1.9	10.7±1.8	- 12.0	20.0	17.8	16.6	2.0	2.5-500ng/mL (plasma)
	100	105±16.8	108±12.3	110±12.5	10.2	16.0	11.4	11.4		
	500	482±55.9	491±86.4	498±43.6	- 0.33	11.6	17.6	17.6		
4-OHDIC	12.5	10.8±2.0	10.2±1.6	9.40±1.4	- 13.4	18.7	15.7	14.8	5.0	5.0-500ng/mL (plasma)
	100	105±13.5	109±17.2	113±4.3	13.0	12.9	15.8	3.8		
	500	469±73.2	486±60.3	462±43.6	- 3.1	15.6	12.4	9.4		
DEX	12.5	11.5±1.9	10.9±1.5	11.2±1.6	- 8.0	17.1	14.0	13.9	0.2	2.0-500ng/mL (plasma)
	100	96.1±10.5	105.6±10.7	110±10.3	3.9	10.9	10.1	9.3		
	500	480±33.1	489±60.1	509±62.6	- 1.5	6.9	12.3	12.3		
DOR	12.5	11.5±0.03	11.0±0.5	11.9±0.05	- 8.3	8.5	8.0	9.6	0.2	1.0-200ng/mL (plasma)
	100	99.1±10.7	105±14.7	105±12.3	3.0	10.8	14.0	11.7		
	500	489±41.6	469±53.9	492±24.2	- 3.3	8.5	11.5	4.9		
CHL	12.5	11.2±1.5	11.4±2.1	10.9±1.9	-10.7	13.6	18.3	17.2	2.0	2.5-500ng/mL (plasma)
	100	101±8.79	95.7±17.8	91.3±10.9	- 4.0	8.7	18.6	12.0		
	500	527±59.0	539±51.7	505±48.5	4.7	11.2	9.6	9.6		
6-OHCHL	12.5	10.5±2.0	11.0±2.1	10.9±1.4	- 13.6	19.1	19.5	12.5	5.0	10-500ng/mL (plasma)
	100	82.0±15.3	93.4±9.06	84.9±9.3	- 13.2	18.7	9.7	11.0		
	500	464±64.0	458±62.3	444±63.0	- 8.9	13.8	13.6	14.2		

Table 2-3: Evaluation of recovery efficiency and matrix effect of probes, metabolites and internal standard (n=6)

Compound	QCs (ng/mL)	Recovery (%)		Matrix effect (%)	
		Value	SD	Value	SD
Midazolam	5	89.2	13.1	10.6	5.4
	40	73.5	10.9	19.8	5.6
	200	81.7	12.1	20.2	7.1
1-OH-midazolam	5	94.6	2.6	-5.1	3.5
	40	92.4	1.6	-17.9	9.9
	200	82.9	5.9	-25.4	10.1
Diclofenac	12.5	113.0	19.6	-8.8	2.8
	100	95.7	10.2	-27.5	3.7
	500	106.0	5.2	-32.0	14.9
4-OH-diclofenac	12.5	106.3	12.1	-29.8	11.2
	100	101.0	5.6	-25.9	13.0
	500	93.5	7.0	-17.7	5.9
Dextromethorphan	12.5	86.4	12.5	-1.7	1.0
	100	87.2	16.1	12.5	0.8
	500	93.9	7.7	0.6	0.3
Dextrophan	12.5	92.6	6.7	-37.5	10.1
	100	101.7	7.5	-25.6	12.5
	500	87.5	5.9	-27.8	14.9
Chlorzoxazone	12.5	112.6	17.8	-10.4	6.9
	100	103.1	11.2	3.7	0.8
	500	101.9	10.2	-6.7	3.5
6-OH-chlorzoxazone	12.5	72.5	11.5	-35.2	10.6
	100	77.5	15.9	-12.5	14.2
	500	79.2	17.5	-42.7	11.9
Dextorphan-d3	10			-34.3	7.9
Midazolam-d4	10			17.8	3.6
Chlorzoxazone-d3	50			-19.8	10.4
4-OH-diclofenac-d4	100			-13.1	5.1

Table 2-4: Stability assessment, high, medium and low QCs were evaluated (n=5)

Mean(SD)	QCs (ng/mL)	72-hrs at 4°C RE% (SD)	24-hrs room temp RE% (SD)	2 months -70°C RE% (SD)
MDZ	5	5.1(1.2)	8.4(4.1)	7.3 (1.5)
	40	6.6( 1.1)	4.9(1.3)	8.8(3.9)
	200	3.5(0.55)	3.3(0.9)	6.5(0.31)
1-OHMDZ	5	-0.7(0.17)	11.9(1.1)	3.2(1.2)
	40	14.9(1.7)	7.4(0.68)	10.2(5.8)
	200	11.0(78)	4.7(0.52)	4.9(2.1)
DEX	12.5	11.2(1.0)	13.6(1.5)	-4.5(1.7)
	100	5.1(2.1)	10.6(1.9)	-9.2(3.1)
	500	-8.9(2.2)	4.3 (0.22)	-11.9(2.4)
DOR	12.5	-3.6(0.41)	-10.1(0.41)	2.5(1.0)
	100	9.3(1.9)	-0.7(0.23)	8.4(2.0)
	500	6.6(1.2)	2.1(0.38)	-3.4(0.23)
DIC	12.5	-5.9(1.0)	-10.8(0.95)	9.4(1.0)
	100	6.9(1.3)	5.3(1.1)	8.4(1.9)
	500	7.9(1.2)	14.8(3.2)	-5.8(17.0)
4-OHDIC	12.5	-11.0(2.6)	-1.6(0.18)	6.6(2.6)
	100	14.1(3.2)	2.0(0.13)	11.0(8.5)
	500	3.0(1.6)	-8.4(1.3)	4.2(12)
CHL	12.5	-5.2(2.0)	-14.8(1.3)	2.5(0.5)
	100	9.8(1.3)	-8.2(0.71)	-8.4(3.5)
	500	8.7(0.45)	3.3(0.94)	3.6(2.0)
6-OHCHL	12.5	-10.9 (2.8)	-12.8(3.4)	3.5(1.3)
	100	8.9 (1.6)	-8.5(2.2)	10.9(5.8)
	500	5.4 (0.39)	-6.9(2.6)	8.7(0.55)

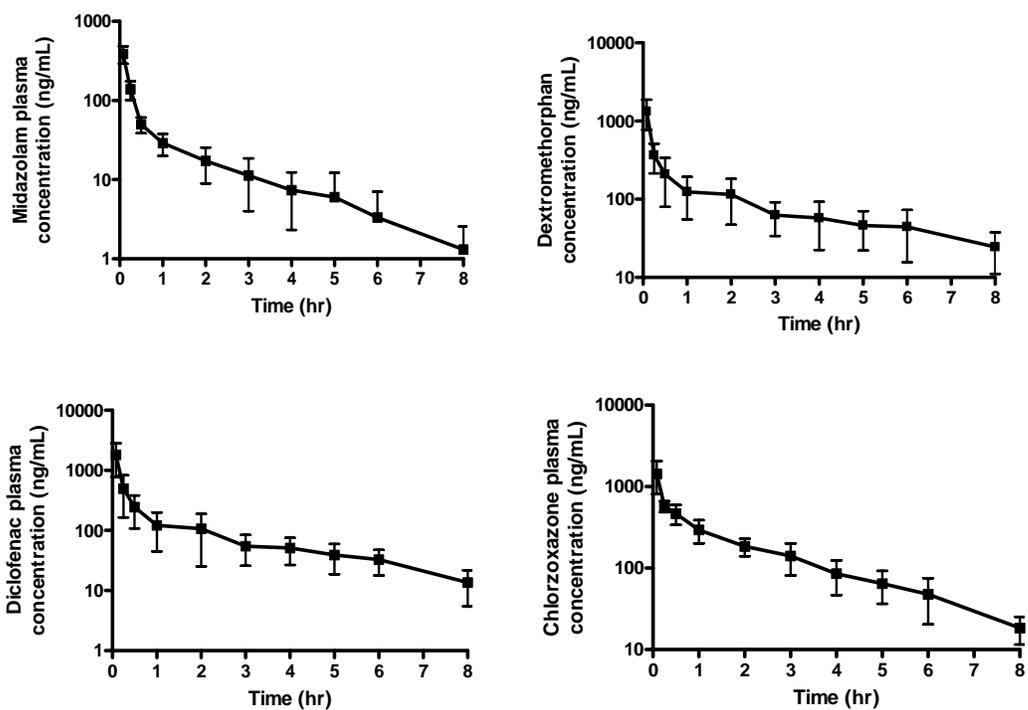


Figure 2-4: Time-plasma concentration profiles of probes after IV bolus of 0.2mg/kg midazolam, 0.5mg/kg diclofenac, 0.5mg/kg dextromethorphan, and 0.5mg/kg chlorzoxazone from six healthy Sprague-Dawley rats.

Table 2-5: Pharmacokinetics parameters estimates from non-compartmental model analysis

(Mean ±SD)

Pharmacokinetic estimates	0.2mg/kg Midazolam	0.5mg/kg Diclofenac	0.5mg/kg Dextromethorphan	0.5mg/kg Chlorazoxazone
AUC <sub>0-T</sub> (ug×min/mL)	9.95 ±1.31	49.3±16.7	53.0±20.2	81.2±16.8
AUC <sub>0-inf</sub> (ug×min/mL)	10.0 ±1.36	51.7±16.7	58.6±21.7	83.7±17.0
C <sub>max</sub> (ng/mL)	388.6 ±95.3	1806±1028	1324±553	1434±624
V <sub>ss</sub> (L/kg)	1.18 ±0.30	1.29±0.61	1.49±0.73	0.65±0.13
T <sub>1/2</sub> (minutes)	74.0±10.5	108.9±36.7	112.8±34.2	77.3±12.0
CLs (mL/min/kg)	20.9±5.94	10.3±2.60	8.63±2.42	6.16±1.15

Table 2-6: Pharmacokinetics parameters of midazolam, diclofenac, detromethorphan and chlorazoxazone estimated from our study and from previous literature when the probe drugs were given in IV/infusion in control rats.

(Mean ±SD)	Author (Reference)	Dose (mg/kg)	CLs (ml/min/kg)	V <sub>ss</sub> /V <sub>d</sub> (L/kg)	T <sub>1/2</sub> (miuntes)
Midazolam	THIS STUDY	0.2	20.9 ±5.9	1.18 ±0.30	74.0±10.5
	(Xia, 2010)	1.0	23.9±5.1	NA	81.1±18.4
	(Strelevitz, 2006)	1.0	22.8	NA	NA
	(Umathe, 2008)	5.0	NA	NA	58.8±3.6
	(Kurosawa, 2009)	5.0	36.6±1.4	1.21±0.07	43.1±3.9
	(Tuk, 2002)	5.0	70±15	3.0±1.0	21.1
Diclofenac	THIS STUDY	0.5	10.3±2.6	1.29±0.61	108.9±36.7
	(Xia, 2010)	0.5	43.5±4.1	NA	110.2±25.8
	(Kim, 2006)	5.0	13.6±1.62	1.58±0.497	175±55.6
	(Reyes-Gordillo, 2007)	3.2	129.21±9.2	0.37±0.04	43.8±4.95
	(Dostalek, 2005)	5.0	NA	0.253±0.081	177±5.0
	(Cui, 2010)	6.75	4.02±0.82	NA	NA
Dextromethorphan	THIS STUDY	0.5	8.63±2.42	1.49±0.73	112.8±34.2
Chlorazoxazone	THIS STUDY	0.5	6.16±1.15	0.65±0.13	77.3±12.0
	(Xia, 2010)	0.5	29.5±5.0	NA	53.2±4.61
	(Muzeeb, 2005)	20	3.17±0.67	0.37±0.13	80.4±15.0
	(Wan, 2006)	25	12.6±1.9	0.403±0.071	22.2±2.6
	(Rockich, 1999)	15	9.23±0.63	0.39±0.04	30.5±1.78
	(Moon, 2003)	20	8.96±3.14	0.373±0.091	32.0±12.6
	(Ahn, 2008)	20	12.3±3.21	0.391±0.061	28.9±2.97

NA: Data not available. To match the same unit, certain units from original literature have been converted

(such as hour to minutes, ml/min to ml/min/kg, etc).

### **2.3.5 Conclusion**

In this study, a method for the simultaneous evaluation of the activities of four major drug-metabolizing cytochrome P450s (CYP3A, CYP2D, CYP2C, and CYP2E) was developed using ultraperformance liquid chromatography-triple quadrupole mass spectrometry (UPLC/MS/MS). UPLC/MS/MS method for analysis all parent drugs and metabolites simultaneously from one injection has been validated with inter-day and intra-day variance less than 20% with extraction recovery greater than 72.5%. The lowest limits of detection ranged from 0.2-5.0ng/mL (1.5-75pg/column). Probe drugs were given to six control rats intravenously at a bolus of 0.2mg/kg midazolam, 0.5 mg/kg diclofenac, 0.5mg/kg dextromethorphan, and 0.5mg/kg chlorzoxazone in a cocktail formulation. The pharmacokinetic estimates obtained from the healthy rats were consistent with previous literature in a different dose range.

## 2.3 DISCUSSION

The UPLC systems takes advantages of sub-2-micron porous particle manufacturing and high pressure hardware, significantly improves the sensitivity and provide high resolution for analysis multiple analytes. In addition, compared to traditional LC column, UPLC column is capable of produce a rapid analysis since it creates wider linear range with high flow rate and high numbers of theoretical plates. Our UPLC/MS/MS method provided the specificity and sensitivity for measuring the concentrations of multiple CYP probes administered via a low-dosed strategy. The method allows us to measure drug and metabolite concentrations in the low picogram range.

Compared with previous cocktails, this study is the only study done using UPLC/MS/MS and provided the in vivo preclinical pharmacokinetic parameters for the probe drugs with a low dose cocktail IV strategy (Zhu et al., 2001; Streetman et al., 2000; Frye et al., 1997). A recent study by Xia et al. has used the very similar dosages as ours (Xia et al., 2010). A previous study by Liu et al., developed a simplified HPLC method and provided the time concentrations profile of five CYP probes in rats, however did not estimate the pharmacokinetic parameters of each probe (Liu et al., 2009). The pharmacokinetic estimates obtained from the healthy rats were consistent with previous literature. This study demonstrated that the ability of using four probe drugs to measure the activity of their respective enzymes was not affected by their coadministration in the current dosage. Therefore, the CYP 3A, CYP2E1, CYP2C and CYP2D activities can be estimated by the cocktail approach in a single experimental session. Future study can be designed upon modifying current bio-analytical method and preclinical animal study protocol such as using alternate probe drug, the dosage, sampling duration, and injection volume. This method is likely to be

particularly useful as a screening, drug drug interaction or phenotyping method for preclinical pharmacological and toxicological research in the future.

In addition, the listed previous pharmacokinetic studies covered different dosages for each probe drug. Therefore, it is possible to extrapolate the pharmacokinetic profiles from a therapeutic dose to a lower dose for a compound characterized by linear PK (Benedetti et al., 2009). Previous study has used LC/MS/MS for microdosing in rats for fluconazole and tolbutamide, which are known to have similar pharmacokinetic properties in humans and rats (Balani et al., 2006). With the analytical method development, the microdosing strategy might be promising for future pharmacokinetic study, especially for early assessment of human pharmacokinetics of new chemical entities (Balani et al., 2006).

Furthermore, it is possible to link and predict preclinical in vivo results from the in vitro animal data with the knowledge of  $K_m$ ,  $V_{max}$ , protein content, liver weight and extraction ratio. It is also possible to extrapolate animal data to pharmacokinetic parameters in humans, with the knowledge of the allometric scaling, the specific equation  $Y = aW^b$  (where the bodyweight ( $W$ ) of the species is plotted against the pharmacokinetic parameter of interest on a log-log scale,  $a$  is compound specific parameter) and molecular properties (Boxenbaum et al, 1995; Mahmood et al., 1999; Jolivet et al., 2005; Mager et al., 2009; Sinha et al., 2011). Therefore, every piece of preclinical data might provide the evidence needed for preclinical to clinical translation.

### 3.0 THE EFFECTS OF THERAPEUTIC HYPOTHERMIA AND CARDIAC ARREST ON SPECIFIC CYTOCHROME P450 ISOFORM ACTIVITY IN RATS

Zhou J, Empey PE, Bies RR, Kochanek PM and Poloyac SM Cardiac arrest and therapeutic hypothermia interaction with isoform-specific Cytochrome P450 drug metabolism

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#### 3.1 INTRODUCTION

As previously discussed, brain injury is a common cause of morbidity and mortality after resuscitation from cardiac arrest (CA) (Geocadin et al., 2008, Manole et al., 2009). Therapeutically reducing body temperature to 32-35°C over 12 to 24 hours after out-of-hospital CA is the proven neuroprotective strategy in these highly susceptible patients (Bernard et al., 2002; Hypothermia after cardiac arrest study group, 2002; Holzer, 2010; Peberdy et al., 2010). Despite the benefit of therapeutic hypothermia, whole body cooling after resuscitation complicates drug therapy due to its potential effects on both drug disposition and response. Previous studies have found significant elevated drug levels and prolonged drug response during hypothermia for drugs such as phenytoin, neostigmine, vecuronium, morphine, propofol, and fentanyl (see detail in Chapter 1). Given the large number of medications used in critically ill patients after CA and possible adverse drug effects, a close pharmacokinetic (PK) and pharmacodynamic (PD) monitoring is required by critical care practitioners (Cullen et al., 1997; Lazarou et al., 1998; Vargas et al., 1998). Currently, the specific changes in drug metabolism and disposition during hypothermia after CA are largely unknown.

Many of the drugs used in CA patients do not have readily measurable pharmacodynamic (PD) endpoints and have narrow therapeutic indices. This fact necessitates the evaluation of a systemic drug metabolism and PK as a tool to ensure drug safety and efficacy. Cytochrome P450 (CYP) enzymes play an essential role in metabolizing medications commonly used in critical care medicine including benzodiazepines, calcium channel blockers, anesthetics, and opioids (Tortorici et al., 2007). The magnitude of altered activity of specific CYP isoforms in CA with changes in body temperature is poorly understood. In addition, as we discussed before, during hypothermia post CA, the alterations in normal drug metabolism, can be quite complex due to suppressed effects from heart failure, multiple organ dysfunction and temperature. Therefore, changes in drug metabolism and drug response may also be a disease state and with interactions of temperature process. Therefore a thorough understanding of both the temperature and cardiac arrest pathophysiologic effects on individual CYP isoform activity is necessary to delineate the alterations in drug disposition.

In this study, we estimated the activities of four major CYP isoforms CYP3A, CYP2C, CYP2D and CYP2E. A probe cocktail of midazolam, diclofenac, dextromethorphan, and chlorzoxazone was utilized for the phenotypic assessment of CYP3A, CYP2C, CYP2D, and CYP2E1 activity, respectively. In vivo validation of all these probe drugs or in combination in cocktail has been previously tested (Scott et al., 1999; Xia, et al., 2010; Yu et al., 2007; Ghassabian et al., 2009; Krösser et al., 2006; Blakey et al., 2004). Midazolam, diclofenac, dextromethorphan and chlorzoxazone were also chosen based on their relative specificity, their relatively short half-life, and their prior clinical use as probe drugs (Frye 2004; Streetman et al., 2000). In addition, these drugs were selected based on the fact that mass spectrometric detection limits allowed for lower

doses of these drugs in rats to decrease the likelihood of probe drug interactions upon co-administration. We hypothesized that the metabolisms of CYP probe substrates decrease during cooling due to the acute effect of hypothermia on the enzyme activity and that these alterations would be isoform specific. The first endpoint of the study is to evaluate systemic clearance of probe drugs within different groups by noncompartmental PK analysis. The secondary endpoint is to determine the specific correlation between hypothermia and CA with probe drug pharmacokinetics by nonlinear mixed effect PK modeling. The objective of this study can be described in Figure 3-1.

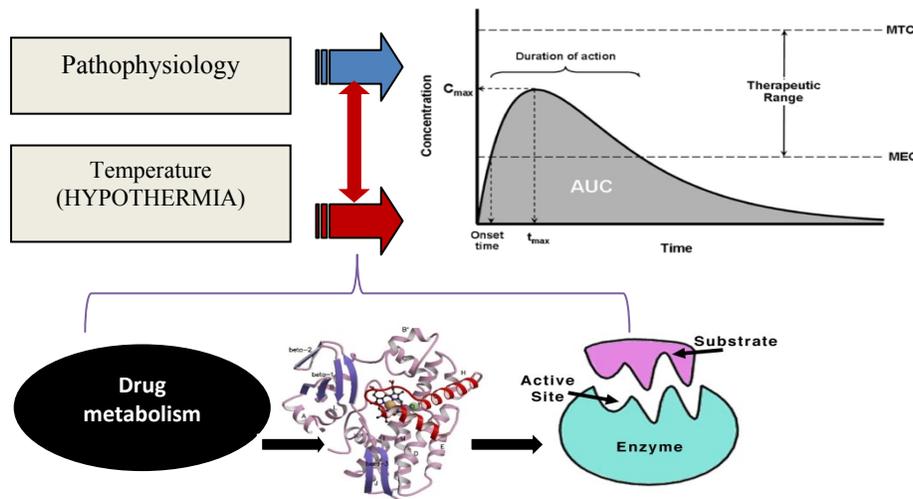


Figure 3-1: The study objectives

## 3.2 EXPERIMENT METHODS AND PROCEDURE

### 3.2.1 Animals

Animal care and all experimental protocols were approved by The University of Pittsburgh Animal Care and Use Committee. Male Sprague-Dawley rats (300-350g) were purchased from Hilltop Laboratories (Scottsdale, PA). The animals were maintained on a 12-h light/dark cycle and were allowed free access to food and water. All animals were acclimated for one week prior to the experiment.

All proposed animal studies were approved by the IACUC and met the standards for humane animal care and used as set by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals. All animals were under isoflurane anesthesia with vecuronium since isoflurane is thought to be less hepatotoxic than halothane or enflurane. Intensive care including saline infusion and necessary bicarbonate adjustment will help decreasing the possibility of distress. In the event that an animal demonstrates undue distress, the animal will be humanely euthanized.

### 3.2.2 Surgery and cardiac arrest procedure

The animals were randomly divided into four groups: (a) sham normothermia, (b) CA normothermia, (c) sham hypothermia, and (d) CA hypothermia (n=6). Rats were anesthetized with 1-1.5% isoflurane via a nose cone, tracheally intubated, and mechanically ventilated to maintain eucapnia (PaCO<sub>2</sub> between 35 and 45 mm Hg) to start surgery and cannulation. Anesthesia was maintained throughout the experiment with isoflurane and oxygen. The left

femoral artery was cannulated for measurement of mean arterial pressure, and the femoral vein was cannulated bilaterally for drug administration and blood sampling. Neuromuscular blockade was induced and maintained with vecuronium 2 mg/kg intravenously before the cannulation. After preparative surgery, isoflurane was washed out with 100% oxygen for 3 minutes and room air for 2 minutes. CA was initiated by asphyxiation via disconnection of the mechanical ventilation. Asphyxial CA was continued for 8 minutes, with approximately 6 minutes of asystole, confirmed by electrocardiogram. Resuscitation was initiated by reconnecting the rat to the ventilator, administering intravenous epinephrine (0.005 mg/kg) and sodium bicarbonate (1mEq/kg), and performing manual chest compressions for about 40-60 seconds until restoration of spontaneous circulation. Sham animals received all of the above surgeries for blood sample collection but were not made hypothermic or subjected to cardiac arrest. Figure 3-2 has shown the cannulation into the blood vein under ScreenLens magnifier. The incision is on the left thigh of the rat. There are two cannulation tubes, one is placed inside of the femoral artery (on the back, already secured on two ends) and the other is placed inside of the femoral vein (on the front, just put in, have not secured the two ends yet). Figure 3-3 has shown that blood pressure change at the time around CA insult (close to zero after disconnection of the intubated tubing) and resuscitation.

### **3.2.3 Hypothermia and temperature measurement**

Hypothermia was induced starting 60 minutes after resuscitation. Systemic hypothermia with a target temperature of 32.5-33°C was initiated by surface cooling for 30 minutes, followed by 15 minutes stabilization. The animals were maintained in a hypothermic state for 8-hr after temperature stabilization. Rectal temperature of the normothermic group was maintained at 37.5-

38°C. Body temperature was continuously measured using a rectal temperature probe. A heating pad, heating light, cooling fan and ice bags were used to adjust and maintain the target temperature.

### **3.2.4 Microdosed formulation**

Microdosed IV cocktail dosage designed at 0.2mg/kg midazolam, 0.5mg/kg dextromethorphan, 0.5mg/kg diclofenac and 0.5 mg/kg chloraxazone. Except midazolam available in injection solution, the other three dextromethorphan, diclofenac and chloraxazone are originally powder. They were dissolved in the saline solution with the minimal ethanol concentration. The final cocktail formulation contains less than 20% (v/v) ethanol in saline. Drug was given to rats intravenously 1.0mL/kg, (e.g. 400g rats, give 0.4mL).

### **3.2.5 Drug administration and pharmacokinetics sampling**

Once rectal temperature was stabilized, the four probe drugs were given intravenously at doses of 0.2mg/kg midazolam, 0.5mg/kg dextromethorphan, 0.5mg/kg diclofenac, and 0.5mg/kg chlorzoxazone simultaneously. The probe substrate cocktail formulation was prepared in saline with ethanol (<20% v/v). Normal saline solution (1.0mL) was injected to flush the catheter after the drug administration. Baseline and blood samples (0.3 mL) at 5, 15, 30, 60, 120, 180, 240, 300, 360 and 480 minutes were obtained via a heparinized syringe. The blood sample volume was replaced with an equal volume of normal saline. The blood samples were centrifuged and the supernatant plasma was transferred to a labeled microcentrifuge tube and immediately placed into a -20°C freezer. Probe drug plasma concentrations or urine metabolites were measured

simultaneously by ultraperformance liquid chromatography- triple quadrupole mass spectrometry (UPLC/MS/MS) which has been described in Chapter 2.

### **3.2.6 Arterial blood gas sampling and physiological parameter**

Multiple arterial blood gas samples (100 $\mu$ L) were collected (at baseline before CA, 10, 30, 60 minutes after return of spontaneous circulation, at the end of hypothermia induction, and every hour during the blood sampling) for the purpose of monitoring the status of the animals over the time. Physiological parameters including pH, MAP, PaCO<sub>2</sub>, pH, lactate, hematocrit, K<sup>+</sup>, Mg<sup>2+</sup>, glucose, and blood urea nitrogen (BUN) were measured at each time point. A maintenance infusion of saline (3mL/hr/kg) was started beginning at 20 minutes after drug administration and continued for 8 hours. Doses of bicarbonate were given as needed to correct acidosis. The complete study procedure has been described in Figure 3-4.

### **3.2.7 Statistical analysis**

Physiological variables were compared by repeated measure ANOVA (among groups and across time) with Bonferroni post hoc correction. Systemic clearances (from the non-compartmental analysis or the population analysis) of each probe drug from different treatments were compared by one-way ANOVA with Bonferroni post hoc. All figures and data expressed as mean  $\pm$  SD as determined from Prism 5 (GraphPad Software, San Diego, CA) or Microsoft Excel. Significance was denoted by a p value <0.05.



Figure 3-2: Cannulation into the blood vein under ScreenLens magnifier. The incision is on the left thigh of the rat. There are two cannulation tubes, one is placed inside of the femoral artery (on the back, already secured on two ends) and the other is placed inside of the femoral vein (on the front, just put in, have not secured the two ends yet).

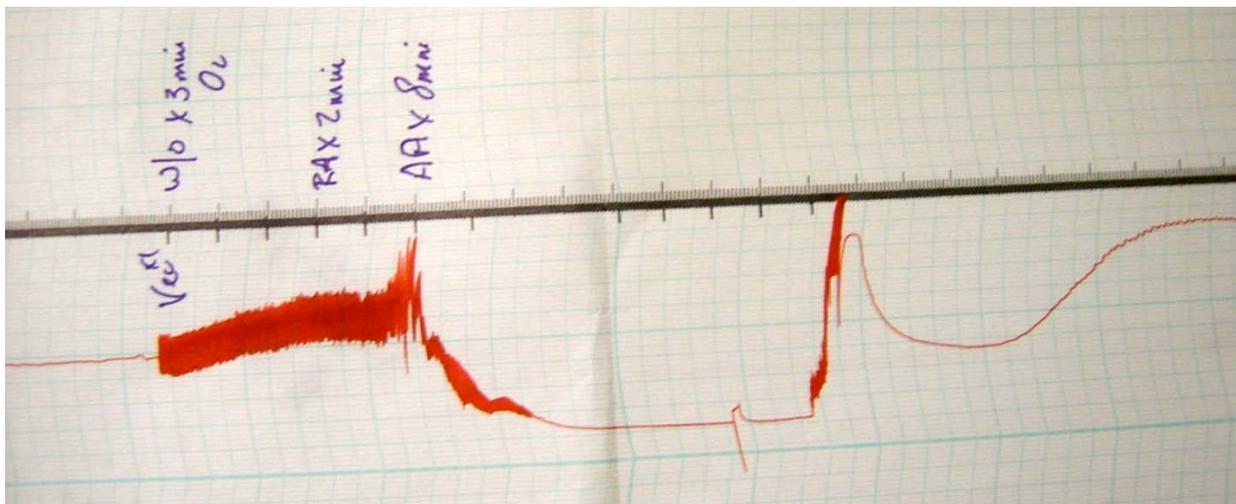


Figure 3-3: Blood pressure curve at the time around CA insult and resuscitation

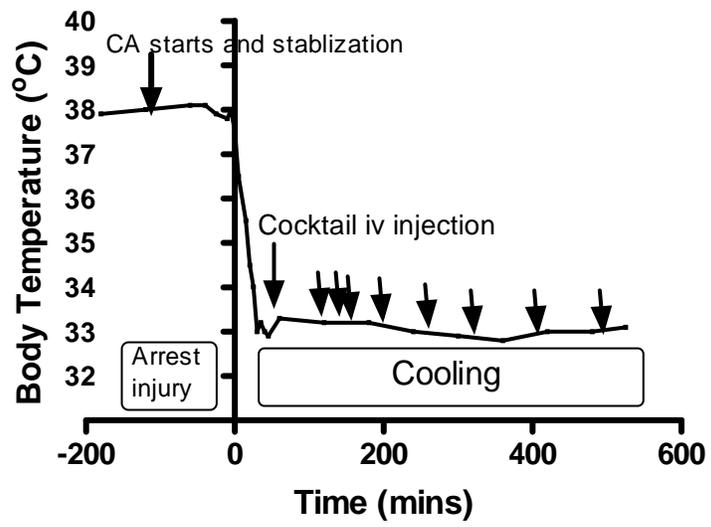


Figure 3-4: Experiment design

### 3.3 PK ANALYSIS

#### 3.3.1 Non-compartmental model analysis and systemic clearances

Non-compartmental pharmacokinetic analysis was used for calculating area under curve ( $AUC_{0-\infty}$ ) and systemic clearance (CL) from each individual time concentration curve of midazolam, diclofenac, dextromethorphan and chlorzoxazone by Winnonlin 5.2. AUC was calculated from drug plasma concentration data based on trapezoidal rule. The clearance then has been calculated from the total dose and  $AUC_{0-\infty}$ .

Non-compartmental pharmacokinetics describes the PK characteristics of a drug without assuming any kinetic compartments. Non-compartmental pharmacokinetics with the method of statistical moments does not describe the concentration-time course of a drug in different body fluids by means different equations. Instead, it calculates PK parameters independently without making assumptions with drug distribution compartments (Gillespie 1991; Cobelli et al., 1984; Rowland et al., 1995). The following parameters that has been calculated by the non-compartmental approach used for evaluation purposes in this thesis:

- 1) Area under the concentration-time curve (AUC): The AUC can be calculated by means of the linear trapezoidal rule.

$$AUC=1/2 \int_{0-t} (C_{k-1}+C_k) \times (t_k-t_{k-1})]$$

where  $t_{k-1}$  and  $t_k$  correspond to two successive measurement time points with the concentrations  $C_{k-1}$  and  $C_k$  during the time period  $t_0- t_k$ . Average concentrations can be obtained by dividing the AUC of one dosing interval by the dosing interval  $\tau$ .

2) Systemic clearance :  $CL = \text{Dose}/AUC$

Clearance by definition represents the volume of the blood cleared through an organ at a unit of time. Clearance is one of the most useful pharmacokinetics parameters. Clearance of a drug from the body depends on the intrinsic ability such as liver and kidney to metabolize or to excrete. For certain drugs that get metabolized primarily, the total clearance is equal to hepatic clearance. In addition, clearance is the function of the blood flow to these organs. For drugs that belong to capacity limited elimination ( $Q \gg CL'_{int}$ ), their clearance highly depend on the hepatic intrinsic clearance. Clearance cannot be estimated from an oral dose because not all the dose gets into the circulation.

$$1) CL_{Total} = CL_H + CL_R, \text{ when } CL_H \gg CL_R, CL_{Total} = CL_H$$

$$2) CL_H = Q \times CL'_{int} / (Q + CL'_{int}), \text{ when } Q \gg CL'_{int}, CL_{total} = CL_H = CL'_{int}$$

### **3.3.2 Population pharmacokinetic (PK) modelling**

#### ***3.3.2.1 Background and importance of population PK***

The subspecialty of population PK was introduced into clinical pharmacology from 1970s as a method to develop model based sparse sampling analysis. Later the population PK has been developed into the discipline of pharmacometrics, pioneered by Sheiner and Beal (Sheiner et al., 1977; Sheiner et al., 1979; Bonate 2005). It describes the typical relations between physiology and pharmacokinetics, the inter-individual, inter-occasion variability in these relations and their residual variability. Population pharmacokinetics seeks to identify the measurable pathophysiologic factors that cause changes in the dose-concentration relationship and the extent

of these changes so that, if such changes are associated with clinically significant shifts in the therapeutic index, dosage can be appropriately modified. The population pharmacokinetic approach has been recommended by guidelines issued by the Food and Drug Administration (FDA) in 1999 and the International Conference on Harmonisation (ICH) as a tool for the identification of the sources and correlates of variability in drug concentrations between individuals representative of those in whom a drug when relevant dosage regimens are administered (Williams and Ette, 2000; Gobburu et al., 2001; Powell et al., 2001). For this thesis research, the major goal of using population PK is to determine whether and to what extent that the factors such as temperature, and disease, leading to the changes in PK and PD response of CYP probe drugs and those clinical relevant drugs.

### ***3.3.2.2 Model estimation method-Maximum likelihood test***

The aim of the PK estimation is to obtain parameters that result in an optimal description of the modeled data given a model function. A likelihood function is a function of the parameters of a statistical model, defined as follows: the likelihood of a set of parameter values given some observed outcomes is equal to the probability of those observed outcomes given those parameter values. The notions of probability and likelihood can be connected together by Bayes' Theorem:  $P(A|B)=[P(B|A)P(A)]/P(B)$ , we can also write:  $P(A)P(B|A)=P(B)P(A|B)$ . If we assign B to be the model and A to be the data, then it will be  $P(\text{data})P(\text{model}|\text{data})=P(\text{model})P(\text{data}|\text{model})$ . After we have the data, and the  $P(\text{data})$  will not change, considering a constant, therefore  $P(\text{model}|\text{data})= P(\text{model})P(\text{data}|\text{model})$ .

The aim of maximum likelihood estimation is to find the parameter value that makes the observed data most likely. When applied to an obtained time concentration data set and given a statically PK model, maximum-likelihood estimation (MLE) provides the estimates for the model's parameters in their maximum likelihood. For example: I am interested in the clearance of a drug from normal healthy human being, but unable to measure the clearance of every single human being in a population. Then I am assuming that the clearances are normally distributed (Gaussian) with some unknown mean and variance, the mean and variance then can be estimated with the MLE approach while only knowing the clearance of some people from the overall population. MLE would accomplish this task by taking the mean and variance as parameters and finding particular parametric values that make the observed results the most possible (given the model). MLE is a method of estimating the parameters of a statistical model used in NONMEM program. In NONMEM, this is done by minimizing the extended least squares objective function value (OFV):

$$\text{OFV}(x_j, \theta, \sigma^2) = \sum_{i=1}^n [\ln \sigma^2 + (y_i - f(x_j, \theta))^2 / \sigma^2]$$

where  $x$  is the independent variable,  $\theta$  is the model parameter,  $\sigma^2$  is the variability, and  $y$  is the vector of data. OFV is equal to  $-2$  of the log likelihood of the fit. Therefore, a minimum OFV reflects the maximum likelihood of parameters that maximize the probability of observing the data given a specific model.

### ***3.3.2.3 Population PK model components***

Nonlinear mixed effect modeling (NONMEM) program is one of the most commonly used programs for population PK modeling and simulation. It was first introduced by Sheiner and

Beal in late 1970 in UCSF. Nonlinear mixed effect modeling approach can be applied to more sparse sampling schedules, allowing a less restrictive and also unbalanced study design. It enables study pooling and the simultaneous investigation of different drug administration routes. In this approach, all parameters are estimated simultaneously, and individual parameters can be determined based on the estimated variances. In addition, mixed effect model allow some or all the parameters to vary with experimental unit through the inclusion of random effects which can flexibly account for the within-unit correlation often observed with repeated measures and provide proper inference (Ette and Williams, 2004). In our study, temperature change correlated to the continuous changing parameters, and therefore it is the best PK estimation approach to calculate the effect of hypothermia on drug metabolism. NONMEM program was used for population PK parameter estimates in this thesis.

#### ***3.3.2.4 Model selection- Statistical comparison***

After model construction, decision on model improvement or deterioration can be based on several criteria. First, the difference in the OFV of two nested models (e.g. between different base models, or between a covariate model and the base model) difference in OFV is approximately  $\chi$ -square distributed. Hence, a difference in OFV of 3.84 and 6.64 points to significant level of 0.05 and 0.01, respectively, given 1 degree of freedom (df). Moreover, precision of parameter estimates can be obtained based on their standard errors. Furthermore, goodness of fit diagnostic plot and visual predictive check are often used to test the robustness and accuracy of the final model (Ette 1997).

### 3.3.3 Population PK model construction in the study

As discussed above, population model components include structure model, pharmacostatistics model (inter-individual, inter-occasion and residual error) and covariate model, which has been shown in Figure 3-5.

#### 3.3.3.1 Structural model and pharmacostatistics model

The population pharmacokinetic model was constructed using a nonlinear mixed-effects approach as implemented in the NONMEM VI program (Icon, Hanover, Maryland). The first order conditional estimation method (FOCE) with interaction was used to estimate all parameters. The population pharmacokinetic model consisted of a pharmacokinetic structural model and a statistical model in which between subject and within subject variability were described. One and two-compartment structural pharmacokinetics models were investigated as base model structures. The one-compartment model structure was evaluated using the ADVAN 1 TRANS 2 subroutine (estimate CL and V) and the two-compartment model structure was implemented as the ADVAN3 TRANS4 subroutine (estimate CL, V1, V2 and Q). The inter-individual variability in the pharmacokinetic parameters was assumed to be log-normally distributed for all population parameters. Inter-individual variability in model parameters are modeled using an exponential term.  $\theta_i = \theta \times e^{\eta_i}$  with  $\eta_i \sim N(0, \omega^2)$ ,  $\theta$  is the population value and  $\eta_i$  is the variable accounting for inter-individual variability with mean zero and variance  $\omega^2$ . Residual variability ( $\sigma^2$ ), the discrepancy between the individual observed ( $C_{obs,ij}$ ) and the individual model-predicted ( $C_{pred,ij}$ ) plasma concentrations, was evaluated using additive, proportional or a combined additive and proportional model  $C_{obs,ij} = C_{pred,ij} \times (1 + \epsilon_{ij1} + \epsilon_{ij2})$ , Where  $C_{obs, ij}$  is the  $j$ th measured observation in individual  $i$ ,  $C_{pred ij}$ , is the  $j$ th model predicted value in

individual  $i$ ,  $\epsilon_{ij}$  is the residual error. Further refinement of the error model was performed as needed.

The model building process was guided by analyzing the goodness of fit plots, plausibility of parameter estimates, precision of parameters estimates, and the lowest objective function value provided by NONMEM. Goodness of fits plots including population predicted concentrations (PRED) vs observed concentration (OBS), individual predicted concentrations (IPRE) vs observed concentration, population predicted concentrations (PRED) vs weighted residuals (WRES) and time vs weighted residuals (WRES) were used for internal validation. Final model parameters were reported with estimates mean with standard error (se%).

### ***3.3.3.2 Covariate model***

After a base model was chosen for each drug, covariate effects on the parameters of that model were evaluated in the following way. Factors treated as categorical covariates were temperature (TEMG, 0= normothermia, 1=hypothermia), and CA injury (CAG, 0= sham, 1=CA). They have been tested in forward addition in either of the following ways:  $P_{TV} = \theta_1 + \theta_2 \times (\text{Cov})$  or  $P_{TV} = \theta_1 \times \theta_2^{(\text{Cov})}$ , where  $\theta_1$  and  $\theta_2$  are estimated fixed effect parameters and Cov is the subject specific value of the categorical covariate. Three physiological parameters (Glucose, BUN and K) were tested in forward fashion as continuous covariates, which has been tested in the following:  $P_{TV} = \theta_1 \times \theta_2^{(\text{Cov}/\text{Medcov})}$ , where  $\theta_1$  and  $\theta_2$  are estimated fixed effect parameters, Cov is the subject specific value of the continuous covariate, and MedCov is the median value of the Cov. To assess whether the model with covariates statistically improved the fit to the data from base model, the difference between their objective function values, referred to as the log likelihood

ratio, was calculated. This ratio is approximately be chi-square ( $\chi^2$ ) distributed. A decrease in the OFV of 3.84 and 6.63 are significant at the  $p=0.05$  and  $p=0.01$  respectively, with 1 degree of freedom. For the final model, stepwise forward addition and then backward elimination process was used. The final covariate models were also evaluated using diagnostic plots, successful minimization, precision for all parameters (standard error %) and visual predictive check. The individual clearances generated for each time point of four groups have been graphed. Full covariate model building process has been depicted in Figure 3-6.

### ***3.3.3.3 Visual predictive check***

Monte Carlo simulations for 1000 times have been performed for each model as predictive check. Monte Carlo simulation performs probability analysis by building models of possible results by substituting a range of values, a probability distribution, for any possible factor that has inherent uncertainty. It then calculates results over and over, each time using a different set of random values from the probability functions. Monte Carlo simulation produces distributions of possible outcome values. Depending upon the number of uncertainties and the ranges specified for them, a Monte Carlo simulation could involve thousands or tens of thousands of recalculations before it is complete (Ette et al., 2007, Nestorov 2001). Therefore, in an internal evaluation procedure 1000 new individual concentration-time profiles were simulated based on the parameter estimates from the final base model. After fitting the final covariate model to each of the resulting datasets, the model parameters were compared with the estimates and the confidence intervals of the original dataset. The predict check of a good final model using simulation should be able to demonstrate that the simulated distribution of median and 95% CI values are in agreement with the observed values. The simulation as well as the goodness of fit

graphs revealed that the final models provided a reliable description of the data with good precision of the structure model estimates and covariate effect.

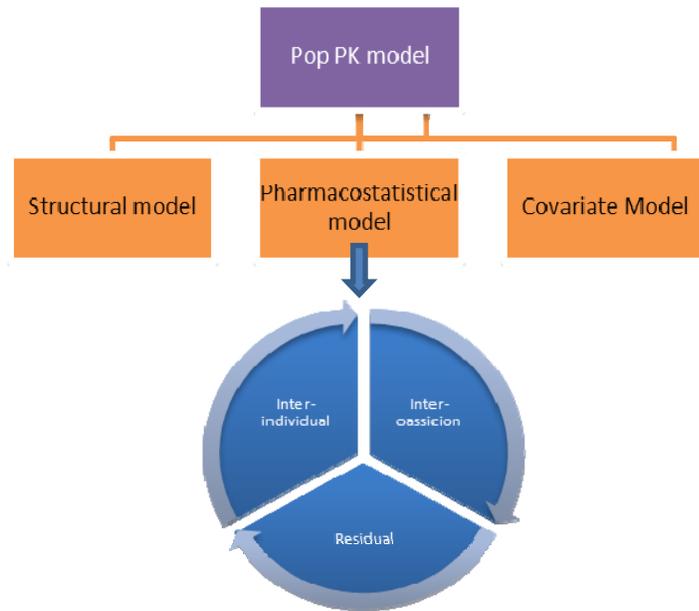


Figure 3-5: Schematic structure of the population PK model

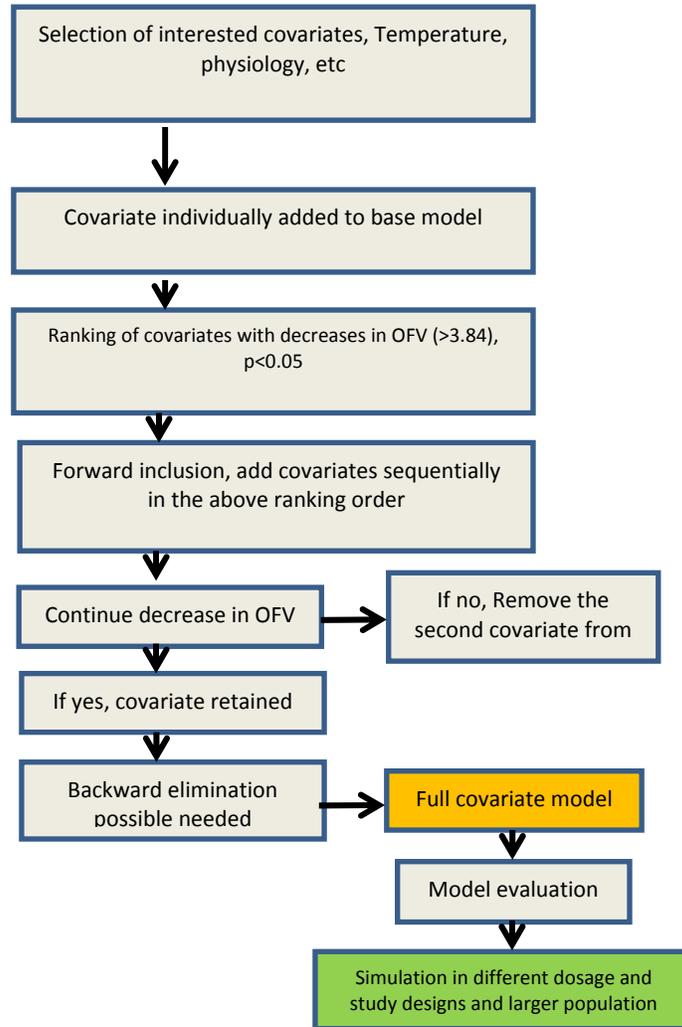


Figure 3-6: Covariate model building process

## 3.4 RESULTS

### 3.4.1 Temperature

The body temperatures at sham normothermia, sham hypothermia, CA normothermia, and CA hypothermic groups were  $37.9\pm 0.4^{\circ}\text{C}$ ,  $32.5\pm 0.4^{\circ}\text{C}$ ,  $37.8\pm 0.5^{\circ}\text{C}$ , and  $32.8\pm 0.3^{\circ}\text{C}$ , respectively (n=6). Time temperature curves of four treatment groups have been depicted in Figure 3-7.

### 3.4.2 Arterial blood gas sampling and physiological parameter

Physiological parameters of MAP, PaCO<sub>2</sub>, pH, glucose, lactate, BUN, hematocrit, Mg<sup>2+</sup> and K<sup>+</sup> from each treatment group at each time point (baseline, 1-hr after resuscitation, end of cooling induction, 4-hr cooling, 8-hr cooling) are shown in Table 3-1. Glucose level decreased over the time in all four groups (p<0.01). BUN increased over the time in all four groups, especially in CA groups. Hematocrit decreased over the time, likely due to multiple blood sampling. Mg<sup>2+</sup> and K<sup>+</sup> both increased at 8-hr in CA groups (p<0.01). At 1-hr after resuscitation, MAP in both CA groups was lower than in the sham control groups. At 1-hr after resuscitation, the glucose level in the CA hypothermia was the lowest among groups, and then recovered. pH in the CA hypothermia group was lower than in the other groups from 1-hr after resuscitation. There was no significant correlation between temperature and multiple physiological parameters (PaCO<sub>2</sub>, glucose, lactate, Hct, BUN and K<sup>+</sup>).

### 3.4.3 Time-concentration curves

Time-concentration curves from 0-8 hrs for midazolam, diclofenac, dextromethorphan and chlorzoxazone were shown in Figure 3-10. Non-compartmental pharmacokinetic analysis was

used for calculating systemic clearance (CL) from each individual time concentration curve of midazolam, diclofenac, dextromethorphan and chlorzoxazone by Winnonlin 5.2 (PharSight, CA). Systemic clearances of each probe drug from different treatments were compared by one-way ANOVA with Bonferroni post hoc test. Significance was denoted by a p value <0.05. All figures and data expressed as mean  $\pm$ SD as determined from Prism 5 (GraphPad Software, San Diego, CA) or Microsoft Excel.

#### **3.4.4 Systemic clearance and non-compartmental analysis**

Systematic clearances calculated from non-compartmental analysis of all four drugs were shown in Figure 3-11. The clearances of midazolam in sham hypothermia, CA normothermia and CA hypothermia groups were 83.2%, 62.4% and 52.5%, respectively, compared to those in sham normothermia group. Significant reduction in the clearance of midazolam in hypothermic CA was found compared with the sham normothermic control ( $681.6 \pm 190.0$  mL/hr/kg vs  $1268.8 \pm 348.9$  mL/hr/kg,  $p < 0.05$ ). In addition, the systemic clearances of midazolam in combined hypothermia groups were 67.8% of those in combined normothermia groups. The clearances of diclofenac in sham hypothermia, CA normothermia and CA hypothermia groups were 89.6%, 102.8% and 75.5% compared to those in sham normothermia group. There was no significant difference found in clearances of diclofenac when compared within four groups.

The clearances of dextromethorphan in sham hypothermia, CA normothermia and CA hypothermia groups were 106.9%, 111.5% and 79.9%, respectively, compared to those in sham normothermia group. There was no significant difference found in clearances of dextromethorphan within four groups. The clearances of chlorzoxazone in sham hypothermia,

CA normothermia and CA hypothermia groups were 63.1%, 45.3% and 40.9%, respectively, compared to those in sham normothermia group. The systemic clearances of chlorzoxazone in combined hypothermia groups were 71.6% of those in combined normothermia groups. Significant reductions in clearances of chlorzoxazone for CA normothermia and CA hypothermia were observed when compared with sham normothermia ( $254.7 \pm 55.5$  mL/hr/kg,  $229.6 \pm 75.6$  mL/hr/kg, vs  $561.8 \pm 215.9$  mL/hr/kg,  $p < 0.05$ ).

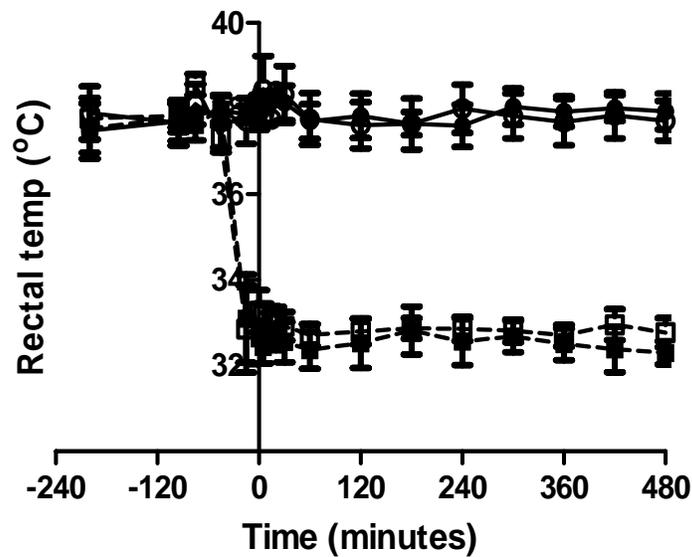


Figure 3-7: Temperature curves of four treatment groups (Mean±SD). Dashed lines are hypothermia groups and solid lines are normothermia groups. (● sham normothermia, ■ sham hypothermia, ○ CA normothermia, □ CA hypothermia).

Table 3-1: Physiological parameters at different time points in four groups

		Baseline before CA	1-hr after ROSC	End of cooling induction, PK baseline	4-hour after cooling	8-hour after cooling
MAP	Sham normo	101.7±7.5	92.7±8.8	102.8±13.8	125.8±16.4	115±15.2
	Sham hypo	110.6±29.5	100.3±11.5	119.5±17.4	131.7±21.9	129.2±14.5
	CA normo	109±27.9	59.3±14.4†	86.3±21.3	110±5.8	136.5±11.9#
	CA hypo	88±11.5	69.2±16.8#†	138.6±12.9#†	130.5±20.5#	126.3±9.9#
pCO2	Sham normo	37.9±5.9	36.4±4.4	35.8±4.2	35.2±3.4	34.8±4.4
	Sham hypo	37.1±4.5	37.2±2.4	40.1±5.1	36.8±7.1	30.8±3.7#
	CA normo	39.3±3.2	32.4±5.4#	36.7±4.1	39.6±8.0	35.4±5.3
	CA hypo	36.5±2.6	29.1±2.3 # †	49.7±5.9#†	42.9±5.5#†	36.9±8.3
Glucose	Sham normo	86.8±20.2	65.7±8.07#	69.7±10.9#	78.2±18.6	75.3±23.5
	Sham hypo	113.5±30.7	76.8±16.6#	87.8±19.1#	83.8±18.2#	89.2±17.4#
	CA normo	112±39.9	63.6±11.8#	56±5.5#†	85.2±28.2#	71±30.3#
	CA hypo	92.6±17.0	53±13.4#†	72.8±29.6#	80.7±16.7#	73.3±38.1#
Lactate	Sham normo	0.78±0.23	0.78±0.29	0.82±0.36	0.32±0.30#	0.5±0.25#
	Sham hypo	0.96±0.24	0.9±0.35	0.56±0.23#	0.62±0.23#†	0.4±0.34#
	CA normo	0.82±0.18	1.40±0.7†	0.7±0.4	0.8±0.23†	1.32±1.5#†
	CA hypo	0.96±0.31	0.88±0.48	0.25±0.13#†	0.47±0.25#†	0.38±0.11#†
BUN	Sham normo	24.7±7.3	22.5±4.5	23.5±6.0	38±12.7#	57.8±19.7#
	Sham hypo	23.2±5.6	24±3.8	23.2±4.6	37.2±10.3#	49.2±16.7#
	CA normo	26.5±8.3	30.1±6.2†	32.3±7.5#†	45±9.8#†	66.8±18.0#†
	CA hypo	26.8±7.9	32.2±9.0#†	31.7±5.7#†	46.2±10.2#†	60.6±16.8#
Hemato crit%	Sham normo	36.7±2.9	35.3±1.2	34.2±1.6	32.3±2.7#	35±1.0
	Sham hypo	35.8±1.6	35.4±2.4	35±1.0	31.6±1.0#	30.8±1.7#†
	CA normo	37±2.8	35.5±1.5	33.3±3.2#	30±1.9#	30.8±1.6#†
	CA hypo	37±4.3	36.8±2.1	37.5±1.6†	31±1.3#	32.8±3.7#†
pH	Sham normo	7.38±0.03	7.38±0.03	7.39±0.03	7.36±0.06	7.36±0.04
	Sham hypo	7.38±0.02	7.45±0.16	7.36±0.01	7.37±0.03	7.39±0.03
	CA normo	7.37±0.03	7.44±0.03	7.39±0.01	7.38±0.02	7.35±0.06
	CA hypo	7.39±0.03	7.43±0.03	7.29±0.04#†	7.32±0.05#†	7.33±0.03#
Mg2+	Sham normo	0.36±0.03	0.37±0.03	0.37±0.04	0.41±0.05	0.41±0.06
	Sham hypo	0.39±0.03	0.39±0.04	0.40±0.03	0.41±0.05	0.43±0.05
	CA normo	0.36±0.04	0.39±0.04	0.41±0.02	0.43±0.04#	0.46±0.06#†
	CA hypo	0.34±0.05	0.43±0.05#†	0.42±0.02#†	0.41±0.05#	0.46±0.09#†
K+	Sham normo	4.75±0.53	4.74±0.40	4.61±0.37	5.25±0.66#	5.62±0.98#
	Sham hypo	4.91±0.30	4.79±0.24	4.12±0.35	3.75±0.34#†	4.83±0.28
	CA normo	4.78±0.35	4.84±0.31	4.84±0.37	5.43±0.47	5.97±0.42#
	CA hypo	4.6±0.48	4.73±0.45	3.89±0.36#†	4.83±0.18	5.67±0.9#

#: p<0.01 compared with the baseline time point within the same treatment group (compare within row), by one way repeated ANOVA. †: p<0.01 compared with the sham normothermia within the same time point (compare within column) by one way repeated ANOVA with Bonferroni post hoc test.

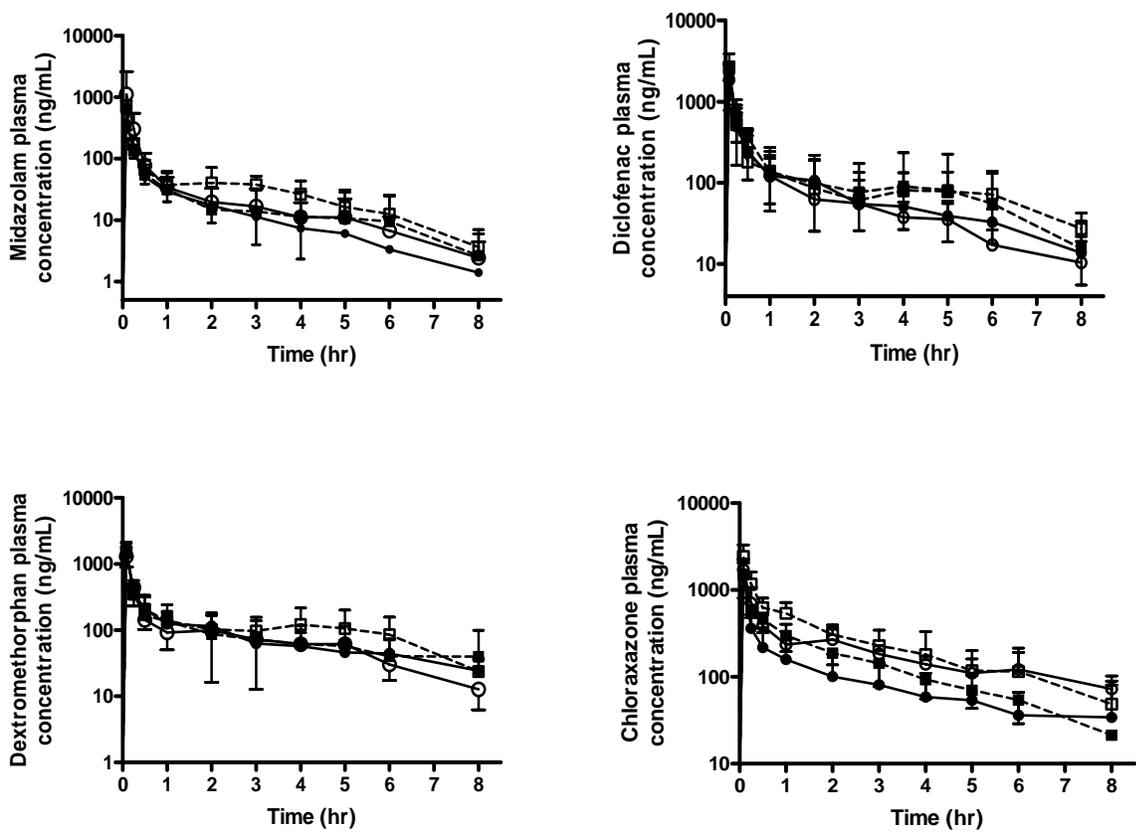


Figure 3-8: Time-plasma concentrations (ng/mL) obtained for midazolam, diclofenac, dextromethorphan and chlorzoxazone from 0-8 hours after IV bolus 0.2 mg/kg midazolam, 0.5 mg/kg diclofenac, 0.5mg/kg dextromethorphan, and 0.5mg/kg chlorzoxazone. Dashed lines are hypothermia groups and solid lines are normothermia groups. (● sham normothermia, ■ sham hypothermia, ○ CA normothermia, □ CA hypothermia).

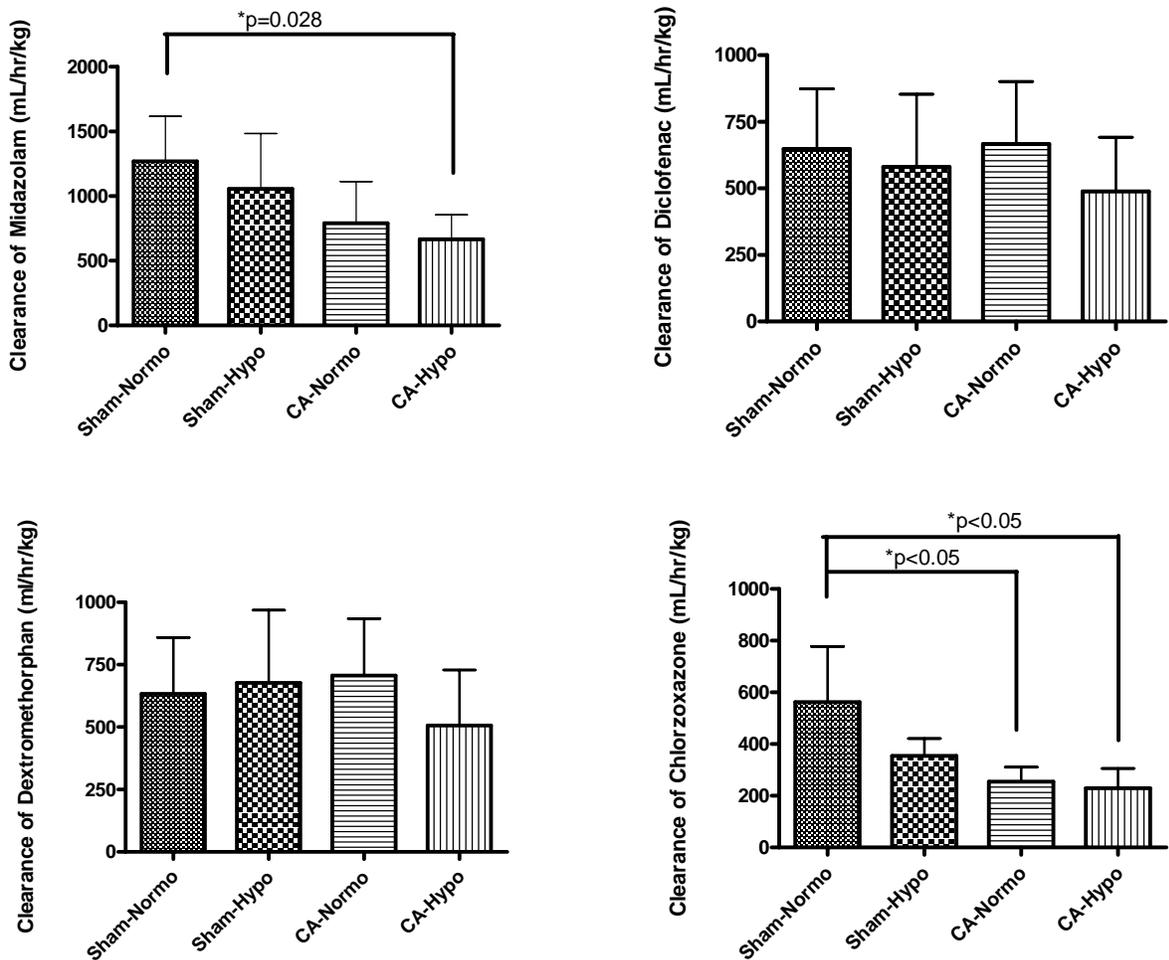


Figure 3-9: Systemic clearances of midazolam, diclofenac, dextromethorphan and chlorzoxazone in four groups by non-compartment model analysis. Significant difference among four groups was tested from one-way ANOVA with Bonferroni post hoc test. \* $p < 0.05$

### 3.4.6 Midazolam population PK model

#### 3.4.6.1 Base Model

The two-compartment model structure was significantly better than a one compartment model for the disposition of midazolam (OFV difference at least 16.0 points, 2df,  $p < 0.001$ ). Normally, in a two-compartment model two different phases can be distinguished in the semi-logarithmic concentration-time plot: a rapid distribution phase and a slower elimination phase whereas the semi-logarithmic concentration-time plot is a linear curve. The plasma concentrations obtained are associated with the central compartment (Figure 3-12). From our time concentration curves for all probe drugs, the two compartment model fits better. The population pharmacokinetic model consisted of a structural model and a pharmaco-statistical model in which between subject and within subject variability were described. Inter-individual variability ( $\omega^2$ ) was chosen for all four CL, V1, Q, and V2 parameters. Residual error model used was described as:  $Y = F + F \times \text{ERR}(1)$ . Parameter estimates of midazolam base model have been listed in Table 4-2.

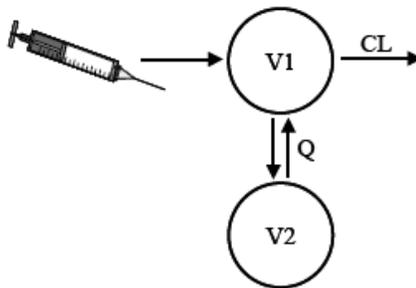


Figure 3-10: Typical two compartment model, in which we chose to estimate CL, V1, V2 and Q.

### ***3.4.6.2 Covariate model***

Two significant covariates were identified reducing the OFV 10.4 points ( $p < 0.01$ ) compared to base model of midazolam. The final pharmacokinetic model for midazolam included a CAG covariate (0=healthy, 1=CA) in systemic clearance (CL), and a TEMG (0=normothermia, 1=hypothermia) covariant in central volume of distribution (V1). CA groups decreased the clearance of midazolam, which can be described as  $CL = 0.990 + CAG \times (-0.318)$ . Systemic clearance of midazolam in CA groups was estimated to be 67.9% of those in healthy groups. In addition, hypothermia decreased the V1 of midazolam, which can be described as  $V1 = 0.258 + TEMG \times (-0.0786)$ . The central volume of distribution of midazolam in hypothermia group was estimated to be 69.5% of those in normothermia. Inter-individual variability of CL, V1, Q and V2 were 11.9%, 1.8%, 46% and 21.4%, respectively.

### ***3.4.6.3 Final model evaluation***

The final covariate model was evaluated using diagnostic plots, successful minimization, precision for all parameters (se%) and a predictive check. Diagnostic plots include individual predicted concentration-observed concentration (IPRED-OBS) and time-weighted residual (TIME-WRERS) of final models of midazolam has been shown in Figure 3-11.

Table 3-2: Population pharmacokinetic parameter estimates (with se%) from NONMEM for midazolam of base model and covariate model.

Parameters Estimate (se%)	Midazolam Base model	Midazolam Final model CL= $\theta_1$ +CAG $\times\theta_5$ ; V1= $\theta_2$ +TEMG $\times\theta_6$
CL( $\theta_1$ )	0.801	0.990 (12.7)
V1( $\theta_2$ )	0.206	0.258 (2.42)
Q( $\theta_3$ )	0.639	0.644(10.6)
V2( $\theta_4$ )	0.991	1.0 (11.9)
Covariate $\theta_5$	—	-0.318(11.5)
Covariate $\theta_6$	—	-0.0786 (16.4)
$\omega^2$ (CL)	0.146	0.119 (6.21)
$\omega^2$ (V1)	0.0075	0.018(14.6)
$\omega^2$ (Q)	0.385	0.460(12.4)
$\omega^2$ (V2)	0.212	0.214 (14.7)
$\sigma^2$	0.112	0.107 (12.5%)
Final OFV	1279.06	1268.8
OFV change		10.4
Covariates p value		p<0.01 (2d.f.)

CL: clearance, L/hr/kg; V1: central compartment of distribution, L/kg; Q: inter-compartmental clearance, L/hr/kg, V2: peripheral compartment of distribution, L/kg. CAG: 0=healthy, 1=CA; TEMG: 0=normothermia, 1=hypothermia;  $\omega^2$ : inter-individual variability;  $\sigma^2$ : residual error

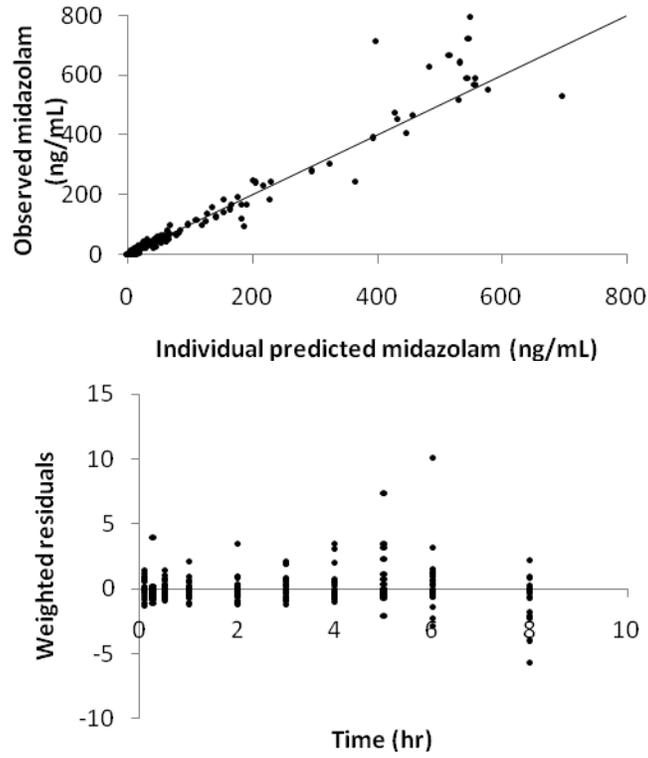


Figure 3-11: Goodness-of-fit diagnostic plots: Individual predicted concentration-Observed concentration (IPRED-OBS) and Time-Weighted Residual (TIME-WRERS) of final models of midazolam. The solid line in the top panel is the line of unity.

### 3.4.6.4 Predictive check

Simulation curves with median curve and 95% CI distribution generated from 1000 times Monte Carlo simulation has been shown below in Figure 3-12. The simulated data and the observed data are in great agreement with each other, suggesting the robust and accuracy of the final model.

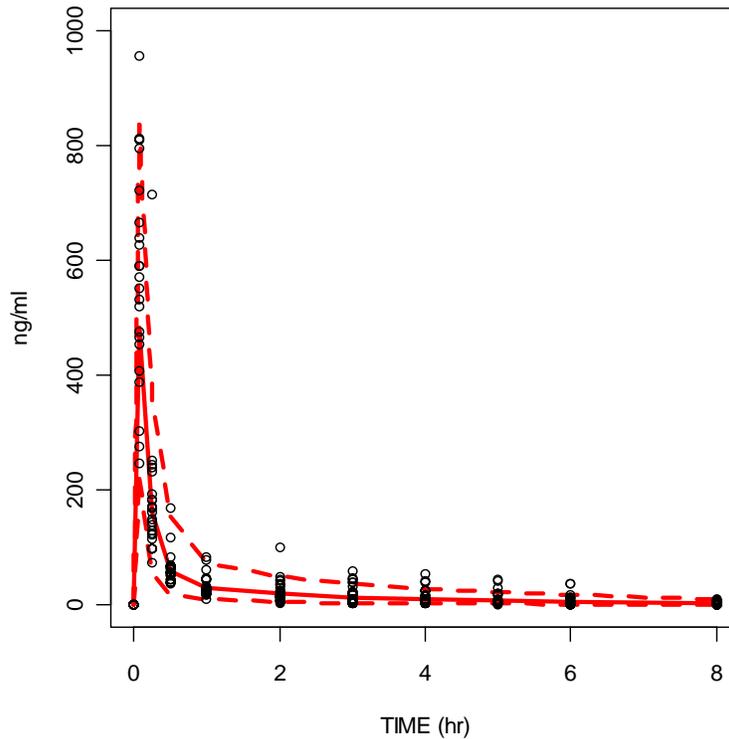


Figure 3-12: Predictive check from 1000 simulations using the final model estimated parameter for midazolam. Solid line is the median of all simulated concentration. Dashed lines are 5<sup>th</sup> and 95<sup>th</sup> percentiles of all simulated values. All dots are observed values from the study.

#### ***3.4.6.5 Discussion of final midazolam model***

Total body clearance for midazolam (CYP3A) during hypothermia after CA was reduced from the clearance of the sham normothermia group as assessed by either the non-compartmental or population model analysis. CA is more likely correlated with the decreased activity of CYP3A observed in the hypothermic CA group. CA and associated sequelae can cause severe metabolic disturbances. Surprisingly, these effects on hepatic drug metabolism are currently also poorly understood.

Previous studies have shown that the metabolism and elimination of drugs are often altered in critically ill patients. Shelly *et al* demonstrated the failure of critically ill patients to metabolize midazolam. The clearance of midazolam was reduced and plasma concentration of its metabolite 1'-hydroxymidazolam was decreased in septic shock patients (Shelly et al., 1987). Spina *et al* has shown that therapeutic monitoring of midazolam in the ICU is warranted (Spina and Ensom, 2007). Kirwan *et al* showed that midazolam can be used to monitor changes in hepatic drug metabolism in critically ill patients. The concentration of midazolam at 4 hours after administration was significantly greater in critically ill patients as compared to normal renal function subjects (Kirwan et al., 2009). A previous study by Vree *et al* has reported the decreased elimination rate of midazolam in the intensive care patients, which may be due to the decreased albumin and altered tissue binding (Vree et al., 1989). The CA effect as well as possible CA-temperature interactions observed in our study thus maybe related to hepatic ischemia, liver dysfunction, compromised renal function, or decreased albumin. The use of hypothermia in patients with CA could thus result in an unrecognized interaction if it is translated to the clinical conditions for predicting metabolism of CYP3A in critical illness.

Previous study by Patal et al. has studied the midazolam PK in patients with congestive heart failure (CHF). In the CHF patients, the elimination half-life of midazolam was prolonged around 25-50% (4-4.5 vs 3 hours), and the systemic clearance of midazolam was reduced to 68% (376 vs 551 ml/min) (Patal, et al. 1990).

The reduced metabolism of midazolam from CA as well as possible CA-temperature interactions observed in our study thus maybe related to hepatic ischemia, liver dysfunction, compromised renal function, or decreased albumin. The use of hypothermia in patients with CA could thus results in an unrecognized interaction if it is translated to the clinical conditions for predicting metabolism of CYP3A in critical illness.

Hypothermia decreased the central compartment volume of midazolam in this study, and therefore also explained the observed higher plasma concentration during hypothermia. Altered pharmacokinetic parameters during hypothermia including the changes of both clearance and volume of distribution have been reported previous. Previous studies demonstrated reduced metabolism of CYP3A substrates during hypothermia (Caldwell et al., 2000; Fritz et al., 2005; Fukuoka et al., 2004; Hostler et al., 2010). In the next chapter, we will discuss one of our clinical studies, in which we have found mild hypothermia reduced midazolam clearance and around 11.1% midazolam clearance decrease per degree Celsius change below 36.5°C in healthy volunteers. Our current understanding is that the combination of hypothermia and CA may both affect the activity of CYP3A and the metabolism and distribution of CYP3A substrates.

### **3.4.7 Diclofenac model**

#### ***3.4.7.1 Base Model***

The two-compartment model structure was chosen. The population pharmacokinetic model consisted of a pharmacokinetic structural model and a statistical model in which between subject and within subject variability were described. Four inter-individual variability ( $\omega^2$ ) were chosen for CL, V1, Q, V2. Residual error model used was  $Y=F+F \times \text{ERR}(1)$ . Parameter estimates of diclofenac base model have been listed in Table 3-3.

#### ***3.4.7.2 Covariate Model***

Final pharmacokinetic model for diclofenac included a TEMG covariate in systemic clearance (CL) ( $p < 0.05$ ), which can be described as  $CL = 0.587 + \text{TEMG} \times (-0.184)$ . The systemic clearance of diclofenac in the combined hypothermic groups was estimated to be 68.7% of those in normothermia groups. Inter-individual variability of CL, V1, Q and V2 were 14.9%, 8.25%, 31.2%, 40.6%, respectively. Final model decreased OFV 6.0 points ( $p < 0.05$ ) from the base model of diclofenac. This effect is considered marginal.

#### ***3.4.7.3 Model Validation***

The final covariate model was evaluated using diagnostic plots, successful minimization, precision for all parameters (se%) and a predictive check. Diagnostic plots for diclofenac has been shown in Figure 3-13.

Table 3-3: Population pharmacokinetic parameter estimates (with se%) from NONMEM for diclofenac.

Parameters Estimate(se%.)	Base model	Final model CL= $\theta_1 + \text{TEMG} \times \theta_5$
CL( $\theta_1$ )	0.494(5.28)	0.587(6.55)
V1( $\theta_2$ )	0.139 (2.06)	0.138 (2.31)
Q( $\theta_3$ )	0.513 (8.28)	0.507 (8.22)
V2( $\theta_4$ )	0.868 (14.0)	0.854 (15.1)
Covariate $\theta_5$	—	-0.184 (9.06)
$\omega^2$ (CL)	0.175 (7.34)	0.149 (5.48)
$\omega^2$ (V1)	0.089(9.02)	0.082(12.4)
$\omega^2$ (Q)	0.294(11.0)	0.312(12.6)
$\omega^2$ (V2)	0.401(12.0)	0.406(11.8)
$\sigma^2$	0.119 (1.65)	0.119 (1.87)
Final OFV	1945.54	1939.5
OFV change		6.0
Covariates p value		p<0.05

CL: clearance, L/hr/kg; V1: central compartment of distribution, L/kg; Q: inter-compartmental clearance, L/hr/kg, V2: peripheral compartment of distribution, L/kg. TEMG: 0=normothermia, 1=hypothermia;  $\omega^2$ : inter-individual variability;  $\sigma^2$ : residual error

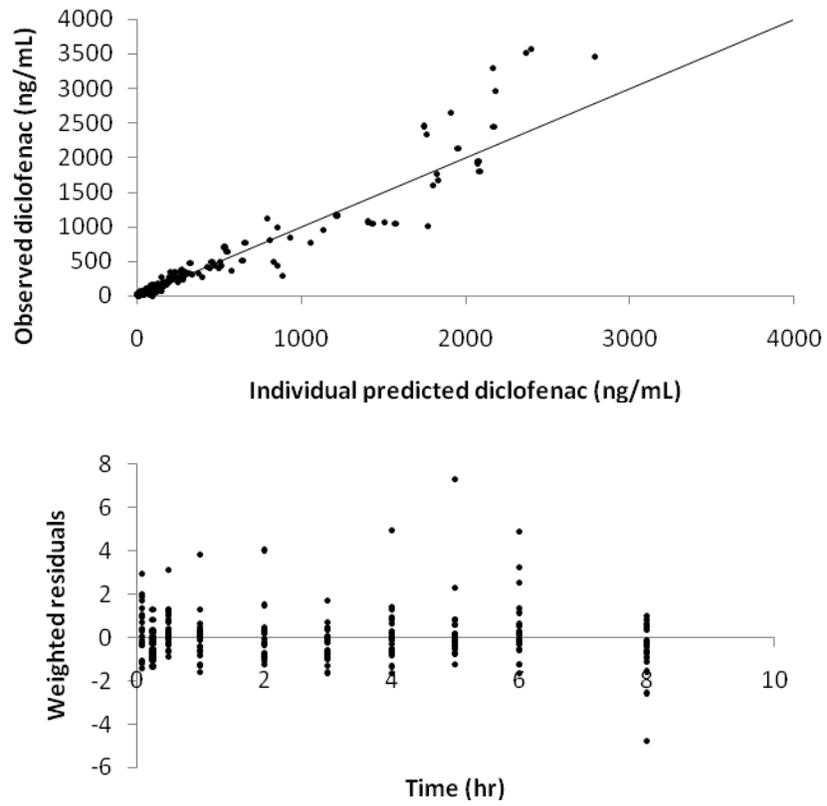


Figure 3-13: Goodness-of-fit plots for diclofenac final model (IPRED-OBS, TIME-WRES).

### 3.4.7.4 Predictive check

Simulation curves with median curve and 95% CI distribution generated from 1000 times Monte Carlo simulation has been shown below in Figure 3-14. The simulated data and the observed data are in great agreement with each other, suggesting the robust and accuracy of the final diclofenac model.

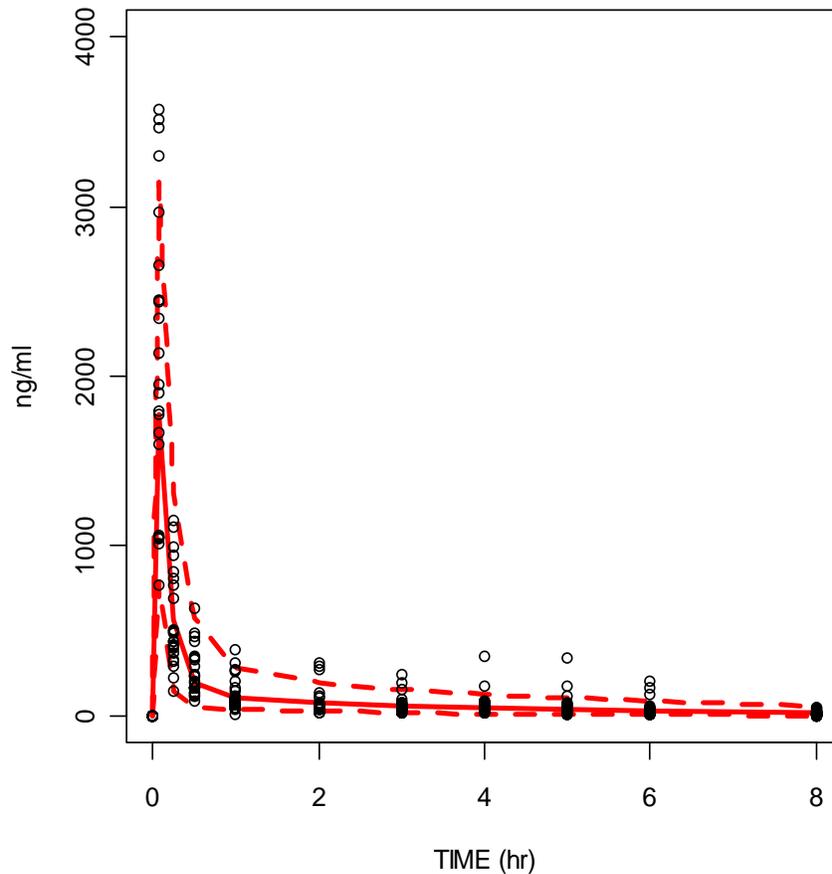


Figure 3-14: Predictive check from 1000 simulations using the final model estimated parameter for diclofenac. Solid line is the median of all simulated concentration. Dashed lines are 5<sup>th</sup> and 95<sup>th</sup> percentiles of all simulated values. All dots are observed values from the study.

#### ***3.4.7.5 Discussion of final diclofenac model***

There was no significant difference found for diclofenac (CYP2C) clearance by non-compartmental model analysis among four groups. However, in the population PK model, two hypothermia groups combined vs two normothermia groups combined, there was a weak association between hypothermia and reduced clearance of diclofenac. Previous studies have shown that hypothermia reduced metabolism of several CYP2C substrates including phenytoin, acenocoumarol and phenobarbital (see details in Chapter 1). Hypothermia increased phenytoin AUC and decreased its clearance in TBI patients (Iida et al., 2001). Clearance of S-acenocoumarol was decreased by 34% at 32.5°C (Daemen et al., 1986). Moderate hypothermia (30-31°C) decreased the metabolism of phenobarbital in critically injured children (Kadar et al., 1982).

In addition, in this population analysis, omega block structure on CL, Q and V2 was used. This indicated a correlation between estimated clearance and estimated volume of distribution. Therefore, this analysis indicated possibilities effects of hypothermia on both diclofenac clearance and on volume of distribution. Therefore, the result from this rat study also referred a differential effect from mathematic calculation and the reduced CYP2C activity, although it may not be clinically significant for comparing temperature effect on CA patients. Based on different patient population and their physiological status, further studies are still needed to confirm the effects of hypothermia in critical ill patients.

### **3.4.8 Dextromethorphan model**

#### ***3.4.8.1 Base Model***

The two-compartment model structure was chosen. NONMEM subroutine ADVAN3 TRANS4 was used which was the same as midazolam and diclofenac models. The population pharmacokinetic model consisted of a pharmacokinetic structural model and a statistical model in which between subject and within subject variability were described. Four inter-individual variability ( $\omega^2$ ) were chosen for CL, V1, Q, V2. Residue error model used for dextromethorphan models was  $Y=F+F \times \text{ERR}(1)$ . Parameter estimates of dextromethorphan base model have been listed in Table 4-4.

#### ***3.4.8.2 Covariate Model***

Final pharmacokinetic model for dextromethorphan included a TEMG covariate in central volume of distribution (V1), which can be described as  $V1=0.204+\text{TEMG} \times (-0.0685)$ . The central volume of distribution of dextromethorphan in hypothermia group was estimated to be 66.4% of those in normothermia. Inter-individual variability of CL, V1, Q and V2 were 26.9%, 17%, 3.85%, and 23.4%, respectively. Final model reduced OFV of 4.7 points ( $p < 0.05$ ) from the base model.

#### ***3.4.8.3 Model evaluation***

The final covariate model was evaluated using diagnostic plots, successful minimization, precision for all parameters (se%) and a predictive check. Diagnostic plots for dextromethorphan has been shown in Figure 3-15.

Table 3-4: Population pharmacokinetic parameter estimates (with se%) from NONMEM for dextromethorphan.

Parameters Estimate(se%.)	Base model	Final V1= $\theta_2$ +TEMG $\times\theta_5$
CL( $\theta_1$ )	0.506(5.67)	0.504 (5.33)
V1( $\theta_2$ )	0.171 (2.02)	0.204 (2.63)
Q( $\theta_3$ )	1.01 (12.7)	1.01 (12.5)
V2( $\theta_4$ )	1.43 (15.2)	1.42 (15.5)
Covariate $\theta_5$	—	-0.0685 (3.2)
$\omega^2$ (CL)	0.269(11.2)	0.269 (11.3)
$\omega^2$ (V1)	0.174 (6.09)	0.170 (5.73)
$\omega^2$ (Q)	0.0609 (5.1)	0.0385 (2.86)
$\omega^2$ (V2)	0.237(9.0)	0.234 (8.99)
$\sigma^2$	0.0827 (1.49)	0.0838 (1.47)
Final OFV	1885.56	1880.9
OFV change		4.7
p value		p<0.05

CL: clearance, L/hr/kg; V1: central compartment of distribution, L/kg; Q: inter-compartmental clearance, L/hr/kg, V2: peripheral compartment of distribution, L/kg. TEMG: 0=normothermia, 1=hypothermia;  $\omega^2$ : inter-individual variability;  $\sigma^2$ : residual error

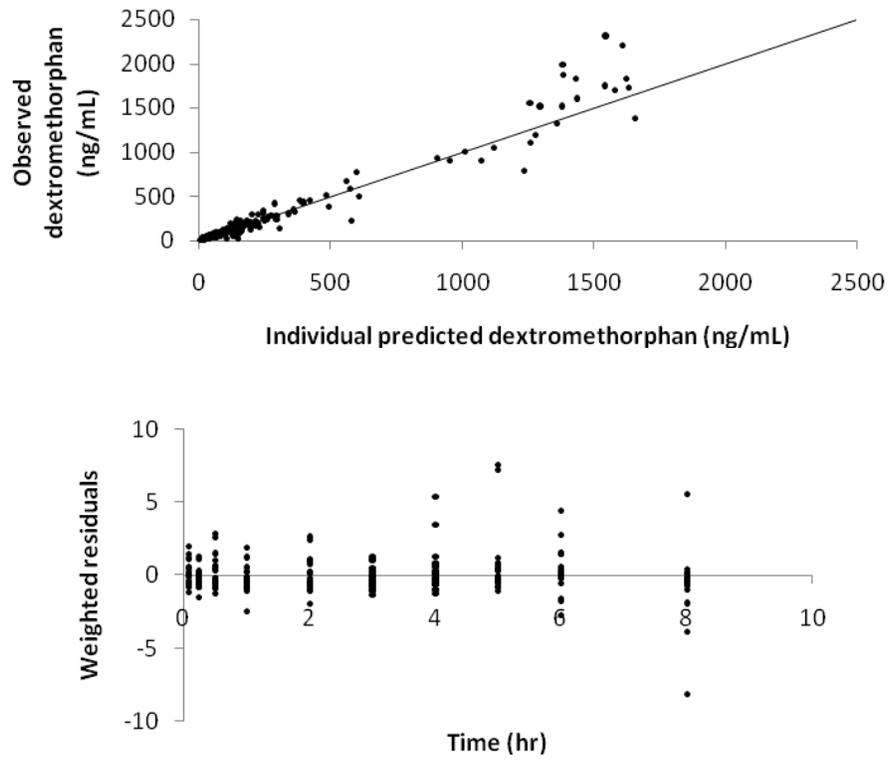


Figure 3-15: Goodness-of-fit plots for dextromethorphan final model (IPRED-OBS, TIME-WRES).

#### 3.4.8.4 Predictive check

Simulation curves with median curve and 95% CI distribution generated from 1000 times Monte Carlo simulation has been shown below in Figure 3-16. The simulated data and the observed data are in great agreement with each other, suggesting the robust and accuracy of the final model.

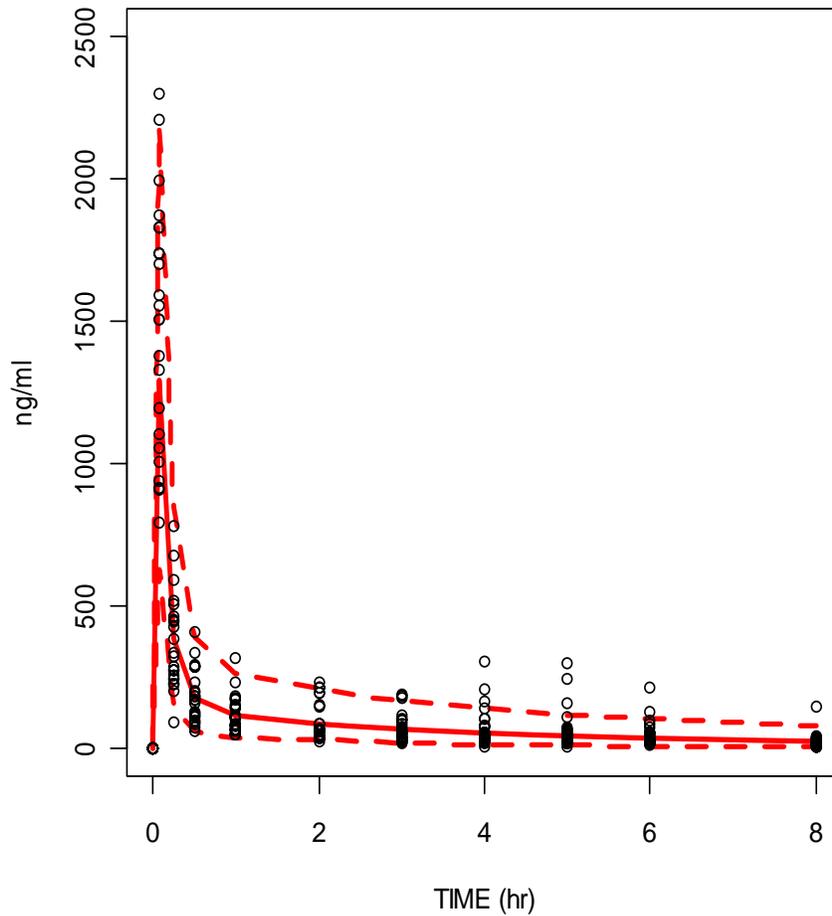


Figure 3-16: Predictive check from 1000 simulations using the final model estimated parameter for dextromethorphan. Solid line is the median of all simulated concentration. Dashed lines are 5<sup>th</sup> and 95<sup>th</sup> percentiles of all simulated values. All dots are observed values from the study.

#### ***3.4.8.5 Discussion of final dextromethorphan model***

There was no significant difference found for clearance of dextromethorphan (CYP2D) among the four groups in the study. Hypothermia was correlated with the reduced central volume of distribution of dextromethorphan. Simulation results showed the concentration at several points at a later time has a large inter-individual variability among all individuals.

Compared with other CYP isoforms, CYP2D as well as CYP2C can interact with specific functional groups in the substrate (e.g., a basic nitrogen for CYP2D6 and an acid for CYP2C9) and help orient them and possibly maintain the metabolic rate and the clearance of the substrates better than other CYP isoforms (Redlich et al., 2008). Therefore, CYP2D may be more stable in the setting of hypothermia and CA injury compared with other CYP isoforms.

During hypothermia, the volume of distribution of many drugs is affected, among which pentobarbital and rocuronium are CYP2D substrates (Kalser et al., 1968; Beaufort et al., 1995; Schaible et al., 1982), which are consistent with this study. In addition, dextromethorphan has been suggested as a medium to high extraction drug, thus its metabolism is majorly related to the blood flow (Miles et al., 2009; Kukanich et al., 2004). Therefore, an alternative CYP2D substrate is needed for further confirmation. Potential mechanisms for altered volume of distribution during hypothermia include global blood perfusion changes, blood pH alterations, altered lipid solubility, and potential alterations in drug tissue binding. Currently no studies have explored the exact mechanisms of these alterations. However, the reduction in volume of distribution has been consistent across studies and warrants future mechanistic evaluation.

### **3.4.9 Chlorzoxazone model**

#### ***3.4.9.1 Base model***

The two-compartment model structure was chosen. The population pharmacokinetic model consisted of a pharmacokinetic structural model and a statistical model in which between subject and within subject variability were described. Inter-individual variabilities ( $\omega^2$ ) were included for CL, Q, and V2 parameters. Residual error model used was  $Y=F+F \times \text{ERR}(1)$ . Parameter estimates of chlorzoxazone base model have been listed in Table 3-5.

#### ***3.4.9.2 Covariate model***

Final pharmacokinetic model for chlorzoxazone included a CAG covariate in systemic clearance (CL), and TEMG covariant in peripheral volume of distribution (V2). CA groups decreased the clearance of chlorzoxazone, which can be described as  $CL=0.433+CAG \times (-0.204)$ . Therefore, systemic clearance of chlorzoxazone in CA groups was estimated to be 52.9% of those in healthy groups. In addition, hypothermia decreased the V2 of chlorzoxazone, which can be described as  $V2=0.970+TEMG \times (-0.478)$ . Therefore, the peripheral compartment volume of chlorzoxazone in hypothermia group was estimated to be 50.7% of those in normothermia. Inter-individual variability of CL, Q and V2 were 12.6%, 21.8%, and 17%, respectively (Table 3-5). Final model reduced OFV of 23.6 points ( $p < 0.01$ ) from base model.

Table 3-5: Population pharmacokinetic parameter estimates (with se%) from NONMEM for chlorzoxazone.

Parameters Estimate(se%)	Base model	Final model CL= $\theta_1 + \text{CAG} * \theta_5$ ; V2= $\theta_4 + \text{TEMG} * \theta_6$
CL( $\theta_1$ )	0.311(3.13)	0.433(5.64)
V1( $\theta_2$ )	0.141 (1.62)	0.142 (1.68)
Q( $\theta_3$ )	0.894(10.7)	0.889(10.6)
V2( $\theta_4$ )	0.697 (8.37)	0.970 (15.1)
Covariate $\theta_5$	—	-0.204 (6)
Covariate $\theta_6$	—	-0.478 (15.8)
$\omega^2$ (CL)	0.224(6.05)	0.126 (3.54)
$\omega^2$ (Q)	0.212(9.39)	0.218 (9.44)
$\omega^2$ (V2)	0.282 (9.41)	0.170 (5.91)
$\sigma^2$	0.0664(0.894)	0.0662 (0.877)
Final OFV	2118.12	2094.5
OFV change		23.6
p value		p<0.01

CL: clearance, L/hr/kg; V1: central compartment of distribution, L/kg; Q: inter-compartmental clearance, L/hr/kg, V2: peripheral compartment of distribution, L/kg. CAG: 0=healthy, 1=CA; TEMG: 0=normothermia, 1=hypothermia;  $\omega^2$ : inter-individual variability;  $\sigma^2$ : residual error

### 3.4.9.3 Model evaluation

The final covariate model was evaluated using diagnostic plots, successful minimization, precision for all parameters (se%) and a predictive check. Diagnostic plots for chlorzoxazone has been shown in Figure 3-17.

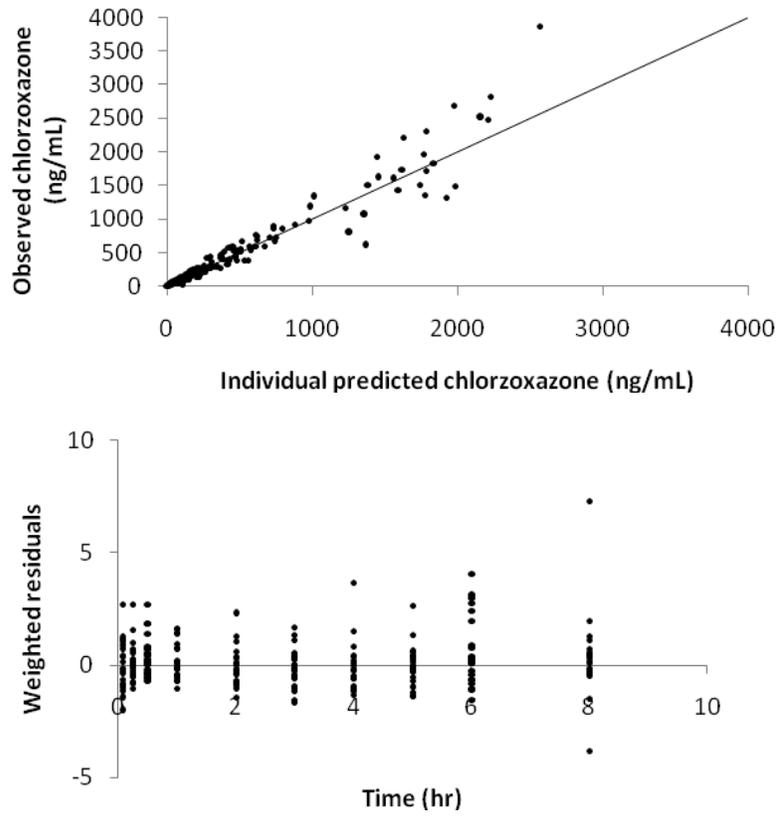


Figure 3-17: Goodness-of-fit plots for chlorzoxazone final model (IPRED-OBS, TIME-WRES).

#### 3.4.9.4 Predictive check

Simulation curves with median curve and 95% CI distribution generated from 1000 times Monte Carlo simulation has been shown below Figure 3-18. The simulated data and the observed data are in great agreement with each other, suggesting the robust and accuracy of the final model.

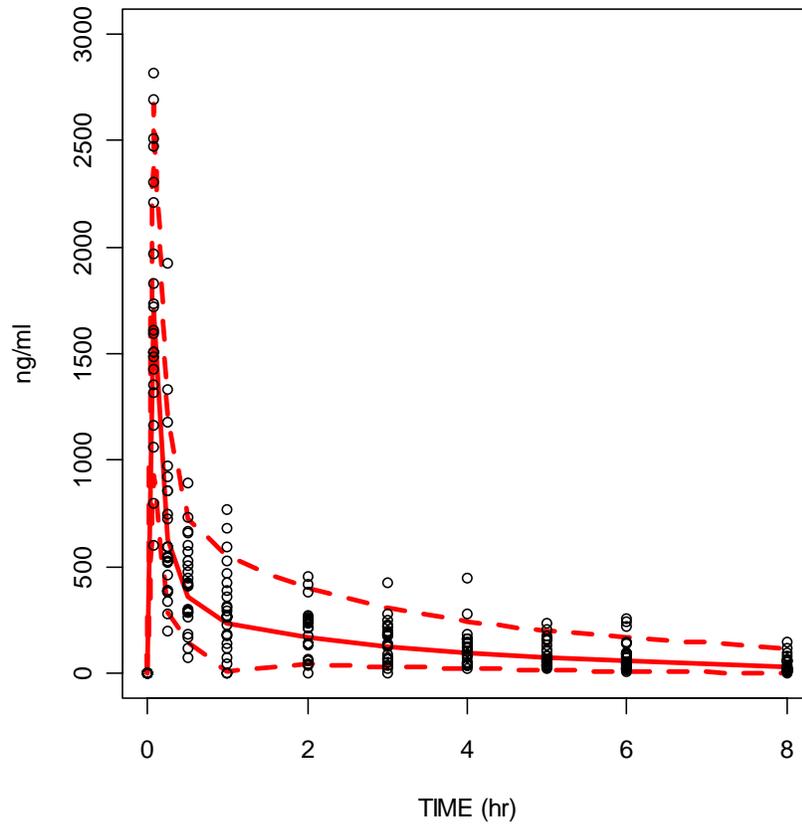


Figure 3-18: Predictive check from 1000 simulations using the final estimated parameter of chlorzoxazone. Solid line is the median of all simulated concentration. Dashed lines are 5<sup>th</sup> and 95<sup>th</sup> percentiles of all simulated values. All dots are observed values from the study.

#### ***3.4.9.5 Discussion of final chlorzoxazone model***

Our results suggest that CYP2E1 activity is affected by both hypothermia and CA injury. There was a hypothermia-CA interaction effect on the clearance of chlorzoxazone (see details in below). In addition, hypothermia reduced the peripheral volume of distribution. Based on our limited numbers of subjects in each group, all population models include midazolam, diclofenac, dextromethorphan and chlorzoxazone should continue test for ETA shrinkage to make sure the estimates described the true distribution.

A previous study by Harbrecht *et al.* showed that the metabolisms of chlorzoxazone and mephenytoin were correlated with the multiple organ dysfunction score and the multiple organ failure score, when they studied CYP2C9, CYP2C19 and CYP2E1 activity in critically ill trauma patients (Harbrecht *et al.*, 2005). Compared with other CYPs, the CYP2E isoform exhibited a relatively constrained heme environment which could be affected easily by a change in physiological conditions. Our previous study showed that hypothermia reduced binding affinity ( $K_m$ ) rather than capacity ( $V_{max}$ ) for chlorzoxazone at 30°C *in vitro*, therefore reduced intrinsic clearance of chlorzoxazone (Tortorici *et al.*, 2006).

#### **3.4.10 Possible CA-hypothermia Interaction**

The individual clearances of midazolam, diclofenac, dextromethorphan and chlorzoxazone from their final models were generated by NONMEM. Temperature-clearance relationship graphs for each drug were shown in Figure 3-19. Temperature-clearance trend lines within sham control and CA groups were added. Possible temperature-CA interaction existed for dextromethorphan and chlorzoxazone when the two trend lines were not parallel with each other (not in the same

trend). The interaction indicated that when both CA and hypothermia exist, the metabolism for dextromethorphan and chlorzoxazone would be different than if either occurred alone.

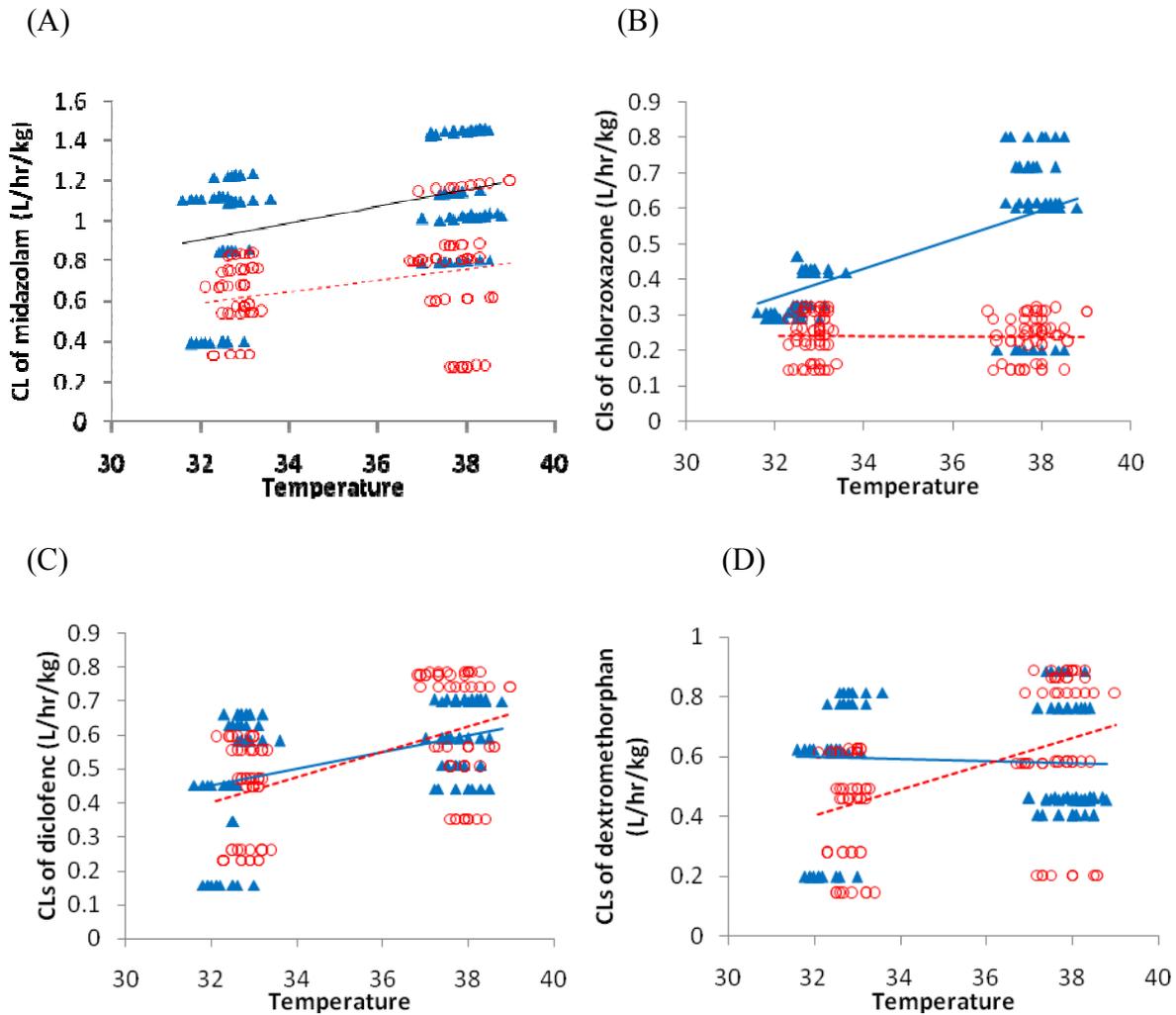


Figure 3-19: Temperature-individual generated clearance from final pharmacokinetic model for midazolam, diclofenac, dextromethorphan and chlorzoxazone. Filled dots ( $\blacktriangle$ ) with solid trend line are healthy rats. Blank dots ( $\circ$ ) with dashed trend line are CA rats.

### **3.4.11 Possible mechanisms for isoform specific effect**

Although the the potential mechanisms for hypothermia CYP isoform specific effect are currently unknown, some of our proposed potential mechanisms including: 1) Specific protein structure and substrate binding affinity: each CYP isoform has its specific quantitative structure activity relationships (QSAR). For example, CYP2E1 is believed to has a very small binding pocket compared with other isoforms, this protein structure can be easily disturbed when environment or physiological changes, therefore leads to the changes of substrate binding affinity and leads to the reduced elimination. CYP2D6 isoform can interact with specific functional groups in the substrate (e.g. a basic nitrogen for CYP2D6) and help orient them and possibility maintain the metabolic rate of the substrates. Our previous experiment in lab showed at lower temperature,  $K_m$  increase for chlorzoxazone. Based on this information and our results, one potential explanation for isoforms specific changes is the affinity change between substrate and the active enzyme binding pocket. 2) Different reaction: different Phase I/CYP reaction may require different level of energy in the stage of product formation and product release from the P450 reaction, and they include: N-dealkylation, aliphatic and aromatic hydroxylation, N-demethylation, O-demethylation, epoxidation, N-oxidation, S-oxidation, etc. Therefore, hypothermia effect could be pathway specific and substrate specific. 3) Reaction cofactors cytochrome b5 and electron transfer: cytochrome b5 is an electron-transport protein, and it is involved in the transfer of the second electron to P450. In vitro system has shown that human CYP3A and CYP2E1, and possibility CYP2C9 and CYP2C19 isoforms require cytochrome b5 for the demonstration of maximal catalytic activities, (but not required for other isoforms) (Shimada et al, 1998). Therefore, a possible change in cytochrome b5 activity would affect the electron transfer in a specific CYP isoform reaction and this matches our observation in this

study. 4) Molecule energy: The rate of CYP mediated metabolism (or clearance) can be correlated with expressions involving either the orbital energies or its equivalent ionisation energy of the substrate molecule. This energy calculated from the substrate molecule itself. The higher the energy of the molecule, the more likely it is a CYP3A4 substrate (Ioannides et al., 2004; Lewis 2003). This orbital energy of the molecule might change under a lower temperature, and therefore, the rate of metabolism will be reduced accordingly and represented in a CYP specific manner. 5) Enzyme-substrate complex and pH environment: Hypothermia leads to a lower pH environment clinically (also showed in my data in the CA hypothermia group, although this may be related to the cardiac arrest acidosis). pH change will re-adjust the original availability of the ionization and un-ionization drugs, and affects the solubility and permeability of the drug. In our study, we observed the clearance reduction in midazolam (CYP3A) and chlorzoxazone (CYP2E1) in the CA hypothermia group from the control, but not for diclofenac (CYP2C) and dextromethorphan (CYP2D). This theory seems to work for diclofenac (pKa 4.0, not affected by small pH change around 7.4) and chlorzoxazone (pKa 8.3, lower pH lowers the un-ionization portion). Therefore, if pH change is an explanation of CYP isoform specific effect, this maybe only a participate mechanism which works along with a combination of other mechanisms.

### 3.5 DISCUSSION

Due to the complex relationship between disease state, temperature alteration, and regulation of CYP functional activity, development of specific dosage guidelines after hypothermic CA has been difficult. This study evaluated the effects of systemic hypothermia mediated alterations on the activity of multiple CYP isoforms in vivo after cardiac arrest. Our results have revealed that the magnitude of the CA and temperature effects are CYP isoform-specific with the greatest alterations in the metabolism observed for CYP3A and CYP2E1 isoforms. Hypothermia reduced volume of distribution of CYP3A, CYP2E and CYP2D probe drugs. This study suggested that the combination of hypothermia with CA was more likely to be associated with decreased enzyme activities. Compared with previous studies focused on single hypothermia effect, the current results have shown the hypothermia isoform specific effect as well as CA injury effect on CYP isoforms. We believe that CA and temperature-CA interaction findings may have clinical implications for patients with CA in whom therapeutic hypothermia is used.

Currently, the population based pharmacokinetic modeling (nonlinear mixed effect modeling) has been used to better describe the complex relationship between multiple covariates (such as diseases, treatment, temperature, drug combination) and drug elimination rate from various clinical and animal studies. This approach provides for the preservation of data structure so that each individual condition contributes to the population description. In addition, fixed effect and random effect are calculated at the same time, therefore, the approach can better capture the mechanism structure while allowing the inter-individual and inter-occasion variability. A previous study by Caldwell et al. reported that hypothermia has a significant association with

inter-compartmental clearances of vecuronium using population pharmacokinetic modeling. A 11.3% clearance decrease per degrees Celsius was predicted for vecuronium (Caldwell et al., 2000). The study by Michelsen et al. demonstrated that the clearance of remifentanyl decreased by 6.37% for each degree Celsius below 37°C in patients who undergoing coronary artery bypass surgery using population pharmacokinetic modeling from NONMEM software (Michelsen et al., 2001). It is one of our goals to develop pharmacokinetic models for each probe drug that can be used to describe the relationship between the changes of temperature and CA injury and metabolism. Understanding biological process and disease progression is essential for further mathematic model estimation and prediction (Bies et al., 2008).

In general, the clearance of hepatically metabolized drugs is either dependent on hepatic blood flow or are dependent on liver enzyme activity / plasma protein binding based on the classic Pang et al. paper describing the well-stirred model (Pang and Rowland, 1977). Since the goal of this study was to evaluate the CYP isoform specific metabolism via the use of probe drugs, all of the selected drugs have low hepatic extraction ratios, therefore, their clearance is highly dependent on enzyme intrinsic activity rather than hepatic blood flow. In addition, the most severe changes in hepatic blood flow would be expected to occur during the acute period of cardiac arrest. Since our drug compounds were not administered until 90 minutes after resuscitation, it is also highly unlikely that hepatic blood flow changes at this later time point would have a significant effect of the hepatic clearance of low hepatic extraction probe drugs. However, the effects of cardiac arrest and hypothermia on hepatic elimination of high and low extraction drugs is important given the significant number of high extraction ratio drugs, such as propofol and fentanyl, that are administered to this patient population. Future studies to elucidate

clearance changes and alterations in hepatic blood flow in these models represent an important area of study.

One limitation of this study is the use of probe drugs as indices of given CYP phenotypic activities. Although the best possible probes were selected for this analysis, it is important to note the significant limitations of the use of these probes as phenotypic measures of CYP isoform specific activity. In particular, diclofenac has been shown in both rats and humans to undergo significant glucuronidation as a major route of elimination. Indeed, several papers have demonstrated that acyl glucuronidation of diclofenac is the predominant route of elimination in non human primates and human liver microsomes (Kumar et al., 2002; Prueksaritanont et al., 2006). In the rat, diclofenac elimination is more dependent on CYP2C, however, glucuronidation is a significant contributor to the overall elimination, therefore, our results with diclofenac are likely due to alterations in CYP2C metabolism and/or acyl glucuronidation. Similarly, other probes have demonstrated involvement of other CYP isoforms and are likely not solely due to changes in a single isoform (Kuo et al., 2003; Wang and Unadkat, 1999). Although these limitations of probe drug specificity exist, the selected drugs do represent different pathways of elimination and therefore, changes in clearance are likely reflective of differing changes in their individual elimination routes. The translational relevance of these alterations to humans will require further investigation with the important consideration that several of these probe drugs are even less selective in humans. It is clear from this study that differential effects are seen with drugs that predominantly rely on differing routes of hepatic metabolism during cardiac arrest and hypothermia.

Collectively, using a rat model, our study reveals important and therefore unrecognized interactions between hypothermia and CA that may magnify the impact seen in CYP drug metabolism caused by either conductor alone. Give the prominent role that the use of mild hypothermia in the management of patients with CA, further translational studies on clinically relevant drugs and pharmacokinetics-pharmacodynamics models are vital to further explain and confirm the isoform specific effect in different disease and temperature states.

## 4.0 MILD HYPOTHERMIA ALTERED THE PHARMACOKINETICS OF MIDAZOLAM IN HEALTHY HUMAN VOLUNTEERS

Hostler D, Zhou J (First two authors contribute equally), Tortorici MA, Bies RR, Rittenberger JC, Empey PE, Kochanek PM, Callaway CW, and Poloyac SM. Mild hypothermia alters the pharmacokinetics of midazolam in normal healthy subjects. *Drug Metabolism and Disposition*, 2010; 38; 5: 781-8

### 4.1 INTRODUCTION

As discussed before, medications commonly used in critical ill patients (antiarrhythmics,  $\beta$ -blockers, calcium channel blockers, benzodiazepines, anesthetics, opioids, anticonvulsants, and proton pump inhibitors, etc) are largely metabolized through CYP450 system. One of the most important CYP isoforms is CYP3A4. Studies to identify the effects of mild hypothermia on CYP450 metabolism in humans are limited and the translational significance of the observations in the rat model remains to be identified. The primary objective of our study was to evaluate the effect of temperature reduction on CYP3A4 activity in healthy human subjects by determining the alterations in the metabolism of midazolam, a well-known CYP3A4 probe in human.

In addition, hypothermia is currently achieved through methods that force core temperature below the internal homeostatic set point. Forced cooling induces compensatory responses such as shivering and vasoconstriction which are an obstacle to reaching target clinical hypothermic temperatures prior to hospital admission (Nagao et al., 2000; Hayashi et al., 2000; Kim et al.,

2009; Polderman and Herold, 2009). Magnesium sulfate ( $MgSO_4$ ) has been shown to facilitate cooling and blunt the shivering response associated with cold saline infusion (Zweifler et al., 2004; Wadhwa et al., 2005). The secondary objective of our study was to determine if benzodiazepine administration with magnesium would facilitate the induction of therapeutic hypothermia in conscious patients. In the current study, we chose a factorial design to examine the effects of administration of cold saline in conjunction with benzodiazepine sedation and magnesium sulfate induced vasodilation as a method to induce hypothermia in concise subjects.

## 4.2 EXPERIMENT METHODS

### **4.2.1 Human subjects**

This study was approved by the University of Pittsburgh Institutional Review Board. Six healthy male subjects between the ages 19-39 years provided informed consent and completed all phases of the study. Each subject received a standard history, physical examination, laboratory studies (serum electrolytes, renal and liver function, thyroid stimulating hormone, hemoglobin, and hematocrit) and a twelve-lead ECG to screen for the presence of cardiac or any other underlying disease. Subjects were excluded if they had an abnormal laboratory value, any known medical problems, or if they were taking any medications with the exception of seasonal allergy medication, over the counter non-steroidal anti-inflammatory drugs, or acetaminophen. Other exclusion criteria included a history of cardiac disease in a family member under the age of 40, allergy to midazolam or other benzodiazepine/narcotic medication, current smoker or recreational drug user, and a body mass index > 35. Body fat percentage was measured by three site skinfold analysis prior to the first protocol visit (Pollack et al., 1980). The subjects were asked not to consume grapefruit juice and herbal dietary supplements including St. John's wort from two days before each study day. Alcoholic drinks and caffeine-containing food and beverages were also not allowed 24 hours before or during the study.

#### ***4.2.1.1 General characteristics***

Subjects were male aged 18 – 39 years. All races and ethnic groups were eligible for enrollment. Eight subjects were screened to ensure six subjects can enter the protocol.

#### ***4.2.1.2 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 did not enroll subjects less than 18 years old.

#### ***4.2.1.3 Inclusion criteria***

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

#### ***4.2.1.4 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. 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

#### ***4.2.1.5 Recruitment procedures***

An announcement has been made in Center for Emergency Medicine, Department of Emergency Medicine, and School of Pharmacy courses and grand rounds. A recruitment flyer has been placed in common areas of the University.

Volunteers have been given a phone number to call to arrange for an appointment with an investigator. Informed consent and screening took place at that meeting. Subjects were 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.

#### ***4.2.1.6 Screening***

A study physician performed a history and physical exam on each subject to assess for the presence of medical conditions. The screening took place in the department of emergency medicine offices on a visit separate from the experimental protocol and normally took approximately 60 minutes. Special focus was 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 was assessed by measuring skinfolds at three sites. Blood samples were collected for sodium, potassium, TSH (thyroid-stimulating hormone), aspartate aminotransferase (AST or SGOT) and alanine aminotransferase (ALT or SGPT), alkaline phosphatase, bilirubin, BUN, creatinine, and a complete blood count to exclude undiagnosed liver, hematologic, and metabolic disease. Finally, a standard 12-lead EKG was 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 were accepted as the normal range. In the event a lab value was outside the normal range, but not clinically significant in the study physician's opinion, the subject was allowed to repeat the lab test once. Two lab tests outside the normal range excluded the subject. Any subject excluded from the study was provided the information concerning their exclusion and they are referred to their primary care physician.

#### ***4.2.1.7 Treatment groups and randomization***

After screening, subjects completed all arms of the study in a randomly generated order. Subjects were entered sequentially into the randomization scheme as they are enrolled. The four treatment groups were 1) 37°C saline infusion (Warm), 2) 37°C saline infusion with magnesium sulfate

infusion (Warm+Mg), 3) saline infusion (Cold), and 4) 4°C saline infusion with magnesium sulfate infusion (Cold+Mg). The four visits were separated by at least one week.

#### **4.2.2 Testing protocol**

We conducted a prospective, randomized, single center, four-way crossover laboratory study. Testing was performed in the Emergency Responder Human Performance Lab under the supervision of a study physician. Subjects were 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 voided their bladder and were weighed.

##### ***4.2.2.1 Induction period***

During this 30-minute period, the subject received a weight based infusion of normal saline and, depending on randomization, a magnesium infusion. Subjects received 30mL/kg of cold (4°C) or (37°C) saline administered by peripheral IV. The total saline dose was delivered over 30 minutes. Subjects received midazolam 2 mg administered intravenously just prior to initiating the infusion. Additional 2 mg doses were administered 10 and 20 minutes into the infusion (total of 6 mg). When randomized to the appropriate arms, 4 g of magnesium sulfate were 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. Normothermia group control was given the warm saline infusion at same speed with or without magnesium. When provided, magnesium was administered with the cold or warm saline infusion.

#### ***4.2.2.2 Maintenance period***

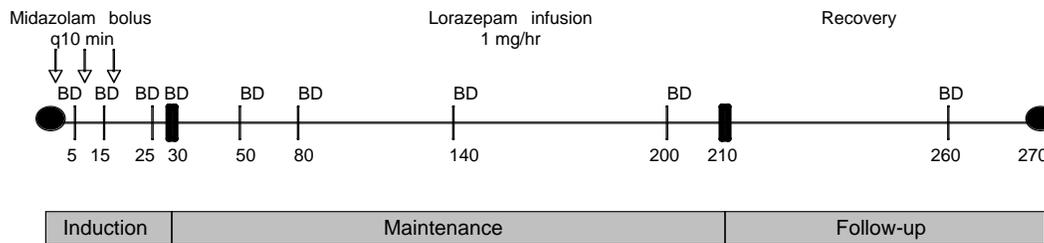
Following the 30-minute saline infusion, an infusion of lorazepam (1 mg/hr) was initiated to maintain mild sedation for three hours. This infusion was titrated to maintain the subject's sedation so that they respond to questions appropriately. Blood samples were drawn and physiological monitoring were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

#### ***4.2.2.3 Follow up period***

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

#### ***4.2.2.4 Monitoring***

Subjects received standard three-lead electrocardiogram, blood pressure monitoring, pulse oximetry, and end-tidal carbon dioxide monitoring throughout each study visit. Blood pressure and subject thermal sensation were measured at five-minute intervals throughout the infusion and every ten minutes thereafter. Our study involved a minimal sedation from given midazolam, a universal Ramsay sedation scale has been used to evaluate the sedation state. Pertaining to this study, ERHPL and has the following equipment available 1) ECG monitor with non-invasive blood pressure, 2) ETCO<sub>2</sub> (end-tidal CO<sub>2</sub>), SPO<sub>2</sub> 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 are present at all procedures. In the event a patient needs to be transferred to Presbyterian Hospital, Pittsburgh EMS will be contacted. The study procedure has been shown in Figure 4-1.



BD= Blood Draw

Figure 4-1: The study procedure. Midazolam was given 3 times bolus at zero, ten, twenty minutes, cold saline infusion from the zero minutes at 30ml/kg until 30 minutes. Mild hypothermia was then maintained by lorazepam infusion and magnesium (when applied). Recovery started from 210 minute and subjects were discharged at 270 minutes. There are 10 time points of blood draws (BD) indicated above.

#### 4.2.3 Temperature measurements

Core body temperature was monitored using a pre-calibrated ingestible thermometer pill that continuously measures temperatures in the range of 0 to 50°C (HQ Technologies, Palmetto, Florida) (Figure 4-2). This pill continuously reads the body temperature while it slowly drifts from esophagus to colon, transmitting the readings to an accompanying handheld. Temperature values recorded by this device are intermediate to esophageal and rectal temperature (O'Brien et al., 1998). The capsule was administered to the subject 60 minutes prior to the beginning of the protocol with approximately 30 mL of water. The protocol was initiated after three consecutive measurements indicated a stable core temperature reading. Skin-surface thermistors were placed over 1) the clavicular head of pectoralis major, 2) supraspinatus, 3) triceps brachii, and 4)

quadriceps femoris muscles. Core and skin surface temperatures were documented every two minutes during the infusion and every ten minutes thereafter. Mean skin temperature (Tsk) was calculated by using the formula  $T_{sk} = \text{chest} (0.25) + \text{back} (0.25) + \text{thigh} (0.3) + \text{arm} (0.2)$  (Ayling, 1986). To mathematically describe the temperature reduction on these subjects, we estimated the area of temperature change as a reflection of cooling burden. Every cooling burden was calculated from individual time-temperature curves. (Trapezoid area from 0-210 minutes minus the area under temperature curve calculated from Prism).



Figure 4-2: Ingestible thermometer pill (copied from HQ.Inc web)

#### **4.2.4 Drug administration and sampling**

Total 6mg midazolam was given intravenously by in 3 separate doses (2mg per dose). The three doses were given at zero, ten, and twenty minutes from starting saline infusion. Blood samples were collected at baseline and at 5, 15, 25, 30, 50, 80, 140, and 200 minutes. The 5, 15, 25, 30, 50, 80, 140 and 200 minutes sample were referring to the time after the first midazolam IV administration. Specifically, the 5 minutes sample and 15 minutes samples were prior to the 2<sup>nd</sup> and 3<sup>rd</sup> midazolam doses, respectively. The blood samples were centrifuged at 2000×g for 10 minutes. Total urine volume was measured at the end of rewarming and a urine aliquot was collected from the final volume for calculating formation clearance of the major metabolite 1'-

hydroxymidazolam. All plasma and urine samples were frozen at  $-70^{\circ}\text{C}$  until the time of analysis.

#### **4.2.5 Drug assay**

Plasma concentrations of midazolam and urine concentration of 1'-hydroxymidazolam were determined by using liquid chromatography-single quadrupole mass spectrometry (LC/MS, ThermoFinnigan single quad MS). A total of  $150\mu\text{L}$  of plasma was added to  $0.5\text{M}$   $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$  ( $\text{pH}=9.6$ ) buffer solution. Samples were then spiked with  $20\mu\text{L}$  of midazolam-d<sub>4</sub> ( $0.1\mu\text{g}/\text{mL}$ ) (Cerilliant Corp, TX) as internal standard. After vortexing,  $5\text{mL}$  methyl-terbutyl-ether was added to the sample, centrifuged at  $3000\times g$ ,  $4^{\circ}\text{C}$  for 10 minutes. The extracted supernatant was collected in a glass tube and evaporated to dryness under gentle nitrogen, followed by reconstitution in  $200\mu\text{L}$  of mobile phase which consisted of  $0.1\%$  formic acid in water and acetonitrile. A volume of  $20\mu\text{L}$  of each sample was injected onto a Thermo RP18  $5\mu\text{m}$ ,  $2.1 \times 150\text{mm}$  HPLC column. The mass spectrometer was operated in the positive ion mode for detection of the protonated-molecular ions  $[\text{M} + \text{H}]^+$  with a cone temperature of  $350^{\circ}\text{C}$  and a capillary voltage of  $2200\text{V}$ . Positive ions at  $m/z$  for midazolam, 1'-hydroxymidazolam and midazolam-d<sub>4</sub> were  $325.9$ ,  $341.9$  and  $330.7$ , respectively. To measure 1'-hydroxymidazolam concentration in the urine, urine samples were incubated with  $2000\text{UI}$  glucuronidase (Sigma, MI) with  $0.2\text{M}$  acetate buffer ( $\text{pH}$   $4.9$ ) at  $37^{\circ}\text{C}$  for 24 hrs. The extraction and LC/MS analytical procedure was similar to the midazolam method described above. Calibration curves ( $r_2 \geq 0.99$ ) for midazolam ( $3\text{-}300\text{ng}/\text{mL}$ ) and 1'-hydroxymidazolam ( $100\text{-}2000\text{ng}/\text{mL}$ ) were linear. Midazolam and 1'-hydroxymidazolam analytical LC/MS methods have been validated with  $< 9.5\%$  and  $< 8.3\%$  inter-day precision, and  $< 7.2\%$  and  $< 7.3\%$  intra-day precision over all

concentrations, respectively. Recoveries of the midazolam and 1'-hydroxymidazolam from liquid-liquid extraction were > 89.2% and > 91.7% over all concentrations, respectively.

#### **4.2.6 Physiological data analyses**

Repeated temperature measurements (core and skin temperatures), heart rate, respiratory rate and end-tidal CO<sub>2</sub> were compared by generalized estimating equations using the factors of time, cold saline infusion, magnesium infusion, and the interaction of cold and magnesium. Estimated effect for each factor is presented as the coefficient estimate (B) with 95% confidence interval (95% CI). Analyses were performed with Prism release 4.0c.

## 4.3 PK ANALYSIS

### 4.3.1 Non-compartmental pharmacokinetic analysis

Non-compartmental analysis (WinNonlin professional Version 4.01, Pharsight, Mountain View, CA) was used to fit the time-concentration data and obtain the estimation for the area under the curve from time zero to infinity ( $AUC_{0-\infty}$ ), area under the curve from time zero to the last sample time ( $AUC_{0-T}$ ), systemic clearance ( $CL_s$ ), estimated half-life ( $T_{1/2}$ ), elimination rate ( $K_e$ ) and volume of distribution ( $V_z$ ). Formation clearance ( $CL_f$ ) of 1'-hydroxymidazolam was calculated by dividing the amount in the urine (1'-hydroxymidazolam concentration in the urine times total urine volume) by the plasma midazolam  $AUC_{0-T}$ . One way repeated ANOVA with Bonferroni post hoc analysis was used to compare formation clearance in the four treatments. Significance was denoted by a p value  $<0.05$ .

### 4.3.2 Population pharmacokinetic modeling

#### 4.3.2.1 Model construction

The population pharmacokinetic model describing midazolam disposition under the conditions outlined in this study was constructed using a nonlinear mixed-effects approach as implemented in the NONMEM V1.1 program (Icon, Hanover MD). The population pharmacokinetics model consists of a pharmacokinetics structural model and a statistical model in which between subject and within subject variability are described. One and two-compartment structural pharmacokinetics models were investigated as base model structures. The one-compartment model structure was evaluated using the ADVAN 1 TRANS 2 routine and the two-compartment model structure was implemented as the ADVAN3 TRANS4 subroutine. The first order

conditional estimation method (FOCE) with interaction was used to estimate all parameters. The inter-individual variability in the pharmacokinetic parameters was assumed to be log normally distributed. The residual variability was evaluated using three candidate model structures, additive, proportional and a combined additive and proportional model  $Y_{ij}=F_{ij} \times (1+Err(1))+Err(2)$ .

The model building process was guided by analyzing the goodness of fit plots, precision of parameters estimates, and the objective function value (OFV) provided by NONMEM. After the base model was selected, we evaluated the effect of core temperature (TEM), heart rate (HR) and magnesium (MG) effect (Y/N) on the pharmacokinetic parameters (CL, V, or CL, V<sub>1</sub>, V<sub>2</sub>, Q). Covariate effects were evaluated using a forward stepwise addition and reverse deletion approach. The impact of the covariates were evaluated using the change in the  $-2 \times \log$  likelihood (-2LL), visual diagnostics, successful minimization, parsimony and physiologic reasonableness of the covariate effects. Improvements in the model were accepted as significant ( $p < 0.01$ , 1d.f.) when a decrease of  $> 6.64$  points in the OFV per added model parameter was observed. Diagnostic plots included population predicted concentrations (PRED) vs observed concentration, individual predicted concentrations (IPRED) vs observed concentration, population predicted concentrations (PRED) vs weighted residuals (WRES) and time vs weighted residuals. Empirical Bayes estimates for individual patient PK parameters clearances were generated and compared with the results from the non-compartmental analysis.

#### ***4.3.2.2 Bootstrap evaluation***

A nonparametric bootstrap approach using sampling with replacement was used to assess the robustness of the model estimates (Parke et al., 1999). Sampling with replacement involved creating a series of datasets of equal size to original data set that are generated by repeatedly sampling individuals from the original dataset, removing these individuals and replacing them at random. The model was refitted to each new data set and this process was repeated 1000 times. The stability of the final model was evaluated by examining the 95% confidence intervals (95% CI) of model parameter estimates. The Wings for NONMEM (G77 Fortran with WFN 408b) implementation for bootstrapping was utilized (Hayes et al., 1989). Bootstrap is considered an important validation tool and model validation is defined as the process of substantiating that the model within its domain of application provides the required functionality, including input and output variable, and that the values it computes are sufficiently accurate for the intended use.

#### ***4.3.2.3 Simulation***

One goal of a PK model is its predictive performance, especially if clinical decision-making will be based upon the model. Simulation has been promoted as a powerful method for predicting the outcome of various drug development scenarios and hence, for designing more cost effective development programs is very useful. The population parameter estimates obtained from the final model were used to simulate the population average concentration-time profiles at three fixed core temperatures, 36.5°C, 34°C and 32°C, over a duration of 400 minutes. Midazolam two mg was administered in the simulation as iv three doses (as used in this study). The simulations were implemented in WinNonlin 4.01 use the population estimated parameters from the NONMEM.

## 4.4 RESULTS

### 4.4.1 Subjects and physiologic variables

Eight subjects provided informed consent and were screened for this study. Two subjects were excluded during screening due to laboratory values that were outside the normal range. The demographics and morphometrics of six subjects were: age  $28.5 \pm 7.6$  (yr), height:  $175.7 \pm 4.7$  (cm), mass:  $78.8 \pm 8.6$  (kg), and body fat:  $17.3 \pm 3.2$  (%). The procedure was well tolerated in all subjects. In our study, we observed relatively minimal sedation with ventilatory and cardiovascular functions unaffected by midazolam. No subject exceeded a value of '3' (Patient awake, responds to commands only) on the Ramsey sedation scale. Patient responds quickly to a voice command and no deeper sedation found. There was an effect of magnesium [B = 8.18 (1.66, 14.7),  $p = 0.014$ ] and on heart rate. There was no effect of cold saline, magnesium, or time on respiratory rate and end-tidal CO<sub>2</sub>.

Since this study is a cross-over design, the intra-individual variability, which is normally smaller than the inter-individual variability was used to estimate a preliminary sample size. Assuming the intra-individual variability is 15%, when  $|\mu_0 - \mu_1| = 25\%$ , based on the power=80% and  $\alpha=0.05$ , per group  $N=6$  has been used in the study.

### 4.4.2 Temperature

Rapid infusion of 37°C saline resulted in a small  $0.4 \pm 0.2^\circ\text{C}$  decrease from baseline. Infusion of 37°C saline with magnesium resulted in a temperature reduction of  $0.9 \pm 0.3^\circ\text{C}$ . Infusion of cold saline reduced core temperature  $1.4 \pm 0.3^\circ\text{C}$  from baseline with a mean nadir temperature of

35.8°C ± 0.3°C. Addition of magnesium to the infusion reduced core temperature 1.8 ± 0.3°C from baseline with the mean nadir at 35.4°C ± 0.4°C. In Cold and Cold+Mg groups, the duration (time below the 36.3°C which is the lowest value in the warm saline infusion groups) of the mild hypothermia is 47.0 ± 24.5min and 101.3 ± 26.6min, respectively. Statistically, there was an effect of cold saline [B = -0.48 (-0.87, -0.92) p = 0.015] and magnesium [B = -0.40 (-0.77, -0.02) p = 0.035] on core temperature. There was neither a time nor a cold saline/magnesium interaction on core temperature. Mean skin temperature changed over time [B = -0.007 (-0.01, 0.0) p = 0.004] but did not differ by the temperature infusion or the addition of magnesium. Cooling burden was calculated and a significant difference was found between Warm and Cold+Mg groups (p=0.01). The core temperature curve and the cooling burden were shown in Figure 4-3 and Figure 4-4.

#### **4.4.3 Midazolam time-concentration profile and non-compartmental analysis**

Six individual time-plasma concentration profiles of midazolam of all four treatments were shown in Figure 4-5. The estimated  $C_{max}$ ,  $AUC_{0-inf}$ ,  $CL_s$ ,  $CL_f$ ,  $T_{1/2}$ ,  $K_e$  and  $V_z$  of the four treatments from noncompartmental analysis was shown in Table 4-1. A significant decrease in the 1'-hydroxymidazolam formation clearance was observed during Cold+Mg compared with Warm group (2.43 ± 0.782 mL/min/kg vs 3.41 ± 0.735 mL/min/kg, p=0.0168). Systemic clearance during Cold+Mg compared with Warm saline group demonstrated a trend towards a significant reduction during hypothermia vs normothermia (3.76 ± 0.386 mL/min/kg vs 4.49 ± 0.560 mL/min/kg, p=0.0568). No significant difference in  $C_{max}$ ,  $T_{1/2}$ ,  $AUC_{0-inf}$ , and  $V_z$  among the four treatments. Individual 1'-hydroxymidazolam formation clearance and midazolam systemic clearance of four treatments were plotted in Figure 4-6.

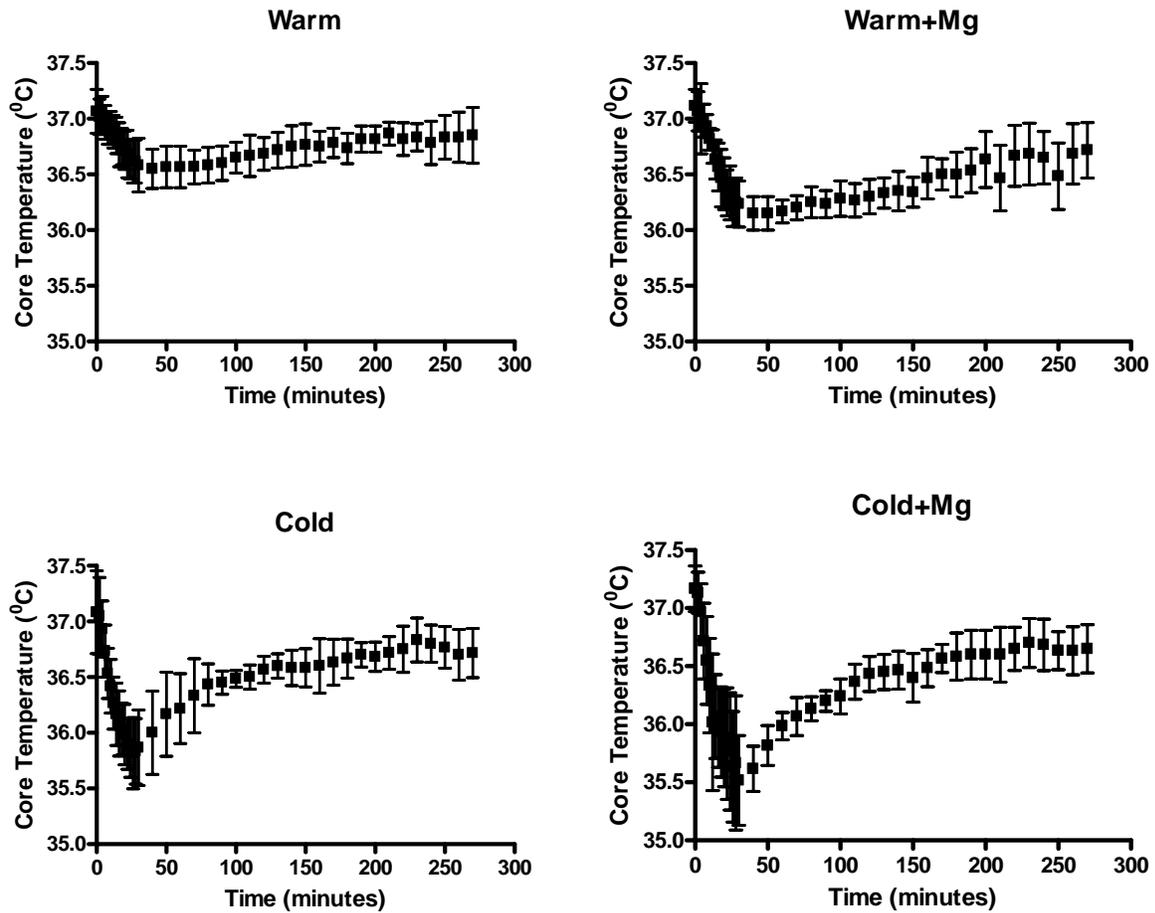


Figure 4-3: Core body temperature (Mean  $\pm$ SD) over time in four treatment groups. The temperatures were recorded every 2 minutes during the first 30 minutes and every 10 minutes thereafter.

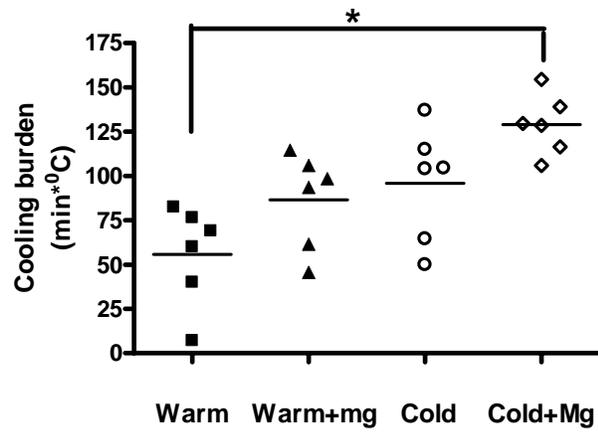


Figure 4-4: Cold burden area was calculated from every individual temperature time curve. There was significant difference found between warm-Cold+Mg groups ( $p < 0.05$ ) of cold burden area.

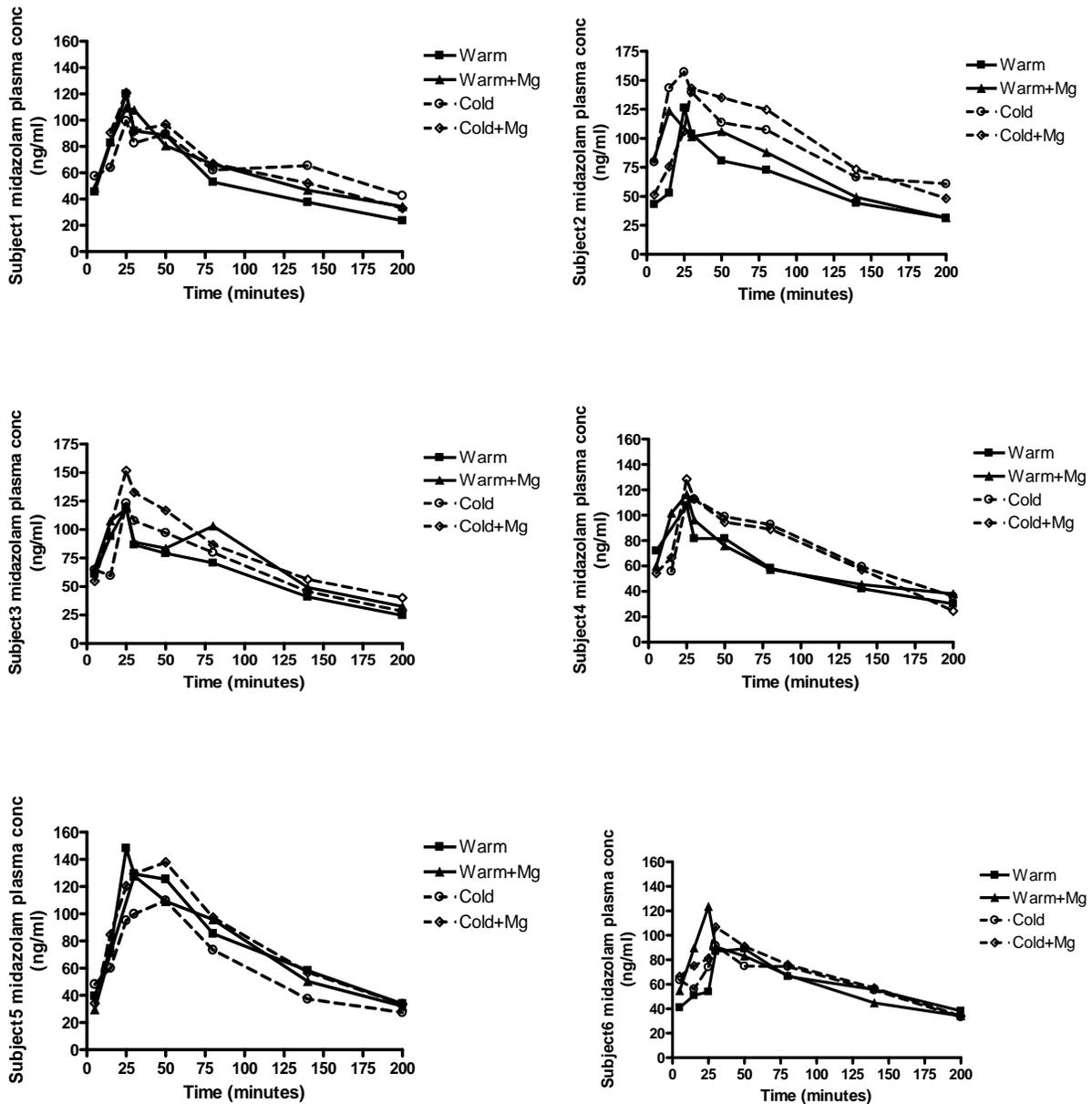


Figure 4-5: Individual time (minute)-concentration (ng/ml) midazolam profile of four treatments. In most subjects, the highest  $C_{max}$  were found in the hypothermia groups (Cold or Cold+Mg group). The concentrations became stably higher after  $T_{max}$  (around 25 or 30 minutes) in hypothermia groups.

#### 4.4.4 Population based nonlinear-mixed effect pharmacokinetic modeling

The data set comprised 258 plasma concentrations from 24 visits. A two-compartment base model structure was significantly better at describing the disposition of midazolam than the one compartment model (OFV difference 16.0 points, 2df,  $p < 0.001$ ). The final two-compartment model including covariate relationships is shown below:  $CL_s \text{ (L/hr)} = 18.5 \times (\text{TEM}/36.5)^{4.24}$ ,  $V_1 \text{ (L)} = 9.11$ ,  $Q \text{ (L/hr)} = 230 \times (\text{TEM}/36.5)^{-17.9}$ ,  $V_2 \text{ (L)} = 37.3 + (\text{HR}/68)^{-5.08} + \text{MG} \times (-3.75)$ . The final model had an objective function value (OFV) of 1152.0, 28.9U lower than the best base model OFV of 1180.9. Graphical model performance is depicted in Figure 4-7. Both systemic clearance and intercompartment clearance of midazolam were affected by core temperature. Temperature was not a significant covariate when tested in relation to the  $V_1$  and  $V_2$  parameters. Heart rate and magnesium significantly affected  $V_2$ . The presence of magnesium reduced the volume of distribution by 3.75L (close to 10% volume of  $V_2$ ). The  $V_2$  changed 73.13% and 75.06% from the maximum to the minimum observed heart rate with or without magnesium, respectively. The pharmacokinetic parameters estimated from the NONMEM analysis were consistent with the non-compartmental analysis. Based on this model, the lowest core temperature 34.8°C in this study would result in a  $CL_s$  of 16.0 L/hr which was 29.6% lower than the clearance from the highest core temperature 37.8°C 22.7L/hr. As temperature decreases, midazolam elimination from the central compartment declines. The model describes an estimated 11.1% reduction in midazolam clearance for every one degree reduction in core temperature from 36.5°C. The individual level model predicted relationships (based on the empirical Bayes estimates) between specific temperature and midazolam clearance are shown in Figure 4-8.

#### 4.4.5 Bootstrapping and simulation

The final model has been assessed further by nonparametric bootstrapping method, the results of bootstrap estimates were consistent with NONMEM estimates which means the final model was relatively stable and robust. NONMEM estimates and the 95% CI from bootstrapping are shown in Table 4-2. A concentration-time profile of midazolam simulated from the population level NONMEM parameters is shown in Figure 4-9. The simulation curve for a core temperature 32 °C has the highest AUC and  $C_{max}$  followed by 34 °C and 36.5 °C. The simulations reflect the model predicted reductions in midazolam clearance at 32°C (a 42.8% reduction compared to 36.5°C) and 34°C (a 26.0% reduction compared to 36.5°C).

Table 4-1: Pharmacokinetic variables of four treatments from non-compartmental analysis

	Warm	Warm/Mg	Cold	Cold/Mg	P value
$C_{max}$ (ng/mL)	123 (23.1)	120 (6.88)	116 (23.0)	132 (16.2)	0.360
$T_{1/2}$ (min)	107 (20.8)	107 ( 20.2)	119 (29.4)	96.3 (9.50)	0.212
AUC <sub>0-T</sub> (µg×min/mL)	13.8 (1.64)	15.1 (1.16)	16.0 (2.90)	17.1 (2.27)	0.0364*
AUC <sub>0-inf</sub> (µg×min/mL)	17.1 (1.63)	18.7 (0.788)	20.8 (4.33)	20.6 (2.33)	0.0649
$V_z$ (L)	54.5 (11.7)	49.8 (9.59)	50.3 (10.6)	41.4 (7.11)	0.0908
$Cl_s$ (mL/min/kg)	4.49 (0.560)	4.12 (0.492)	3.83 (0.881)	3.76 (0.386)	0.0568
$Cl_f$ (mL/min/kg)	3.41 (0.735)	2.65 (0.923)	2.56 (0.917)	2.43 (0.782)	0.0168*

One way repeated ANOVA with Bonferroni post-hoc has been used to detect the difference among the four treatment groups. Data presented as mean (SD). \*p < 0.05

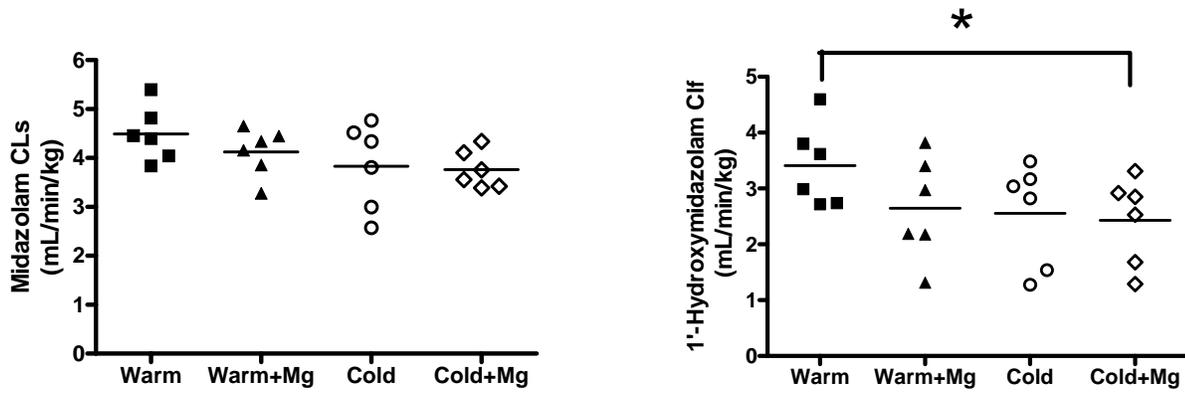


Figure 4-6: The systemic clearance of midazolam and 1-hydroxymidazolam formation clearance from four groups.

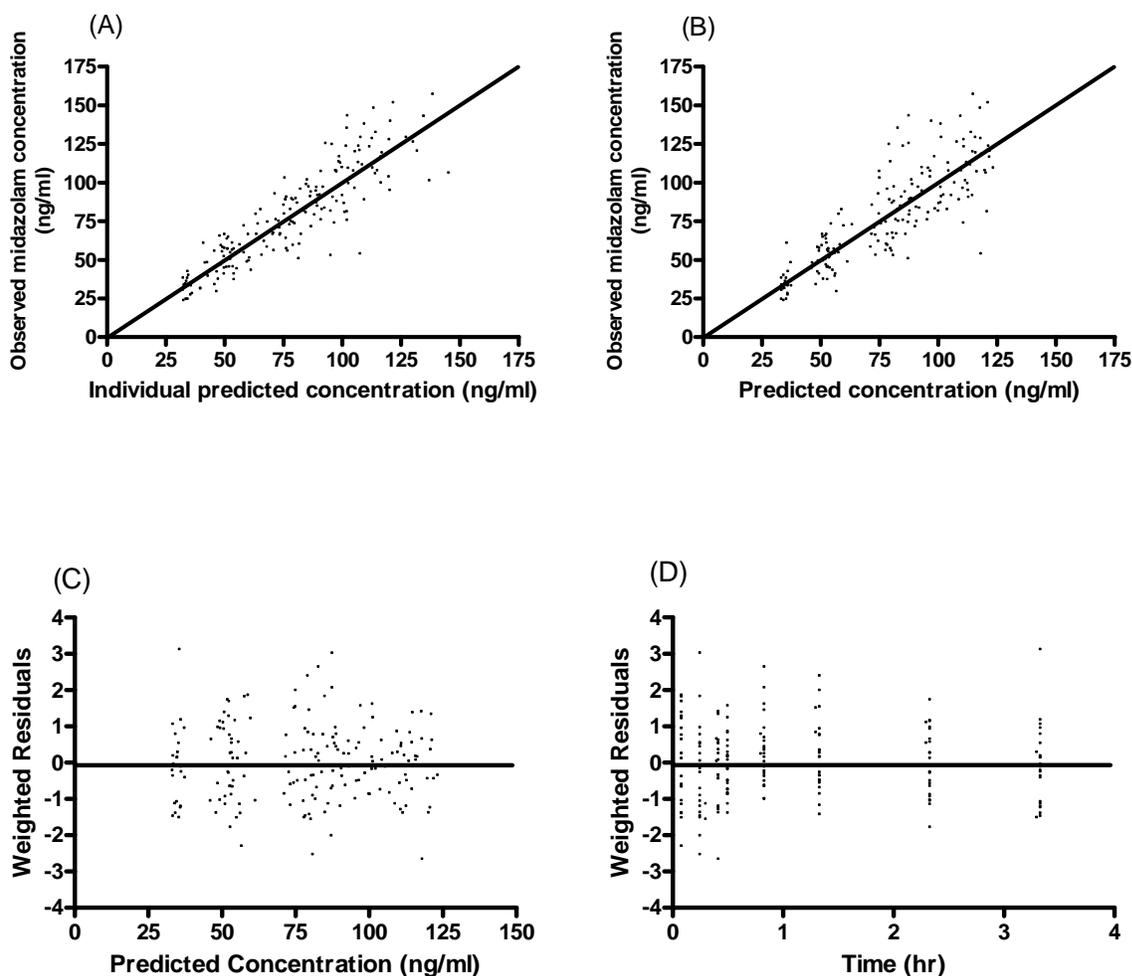


Figure 4-7: Final model diagnosis. Goodness-of-fit plots for the population pharmacokinetic model from NONMEM analysis. Individual predicted concentrations vs observed concentrations, and the population predicted concentrations vs observed concentrations. The straight lines are the lines of unity (A-B). Population predicted concentrations vs weighted residue and Time vs weighted residue (C-D).

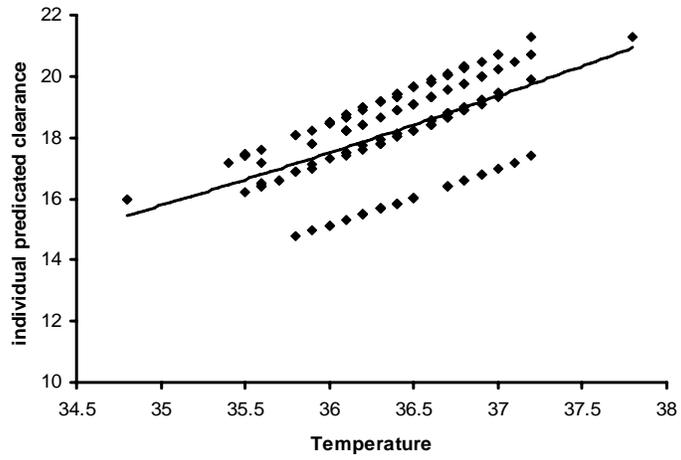


Figure 4-8: The relationship between core body temperature and individual systematic clearance (L/hr) from NONMEM estimation. With temperature decreases, the midazolam systematic clearance decreases.

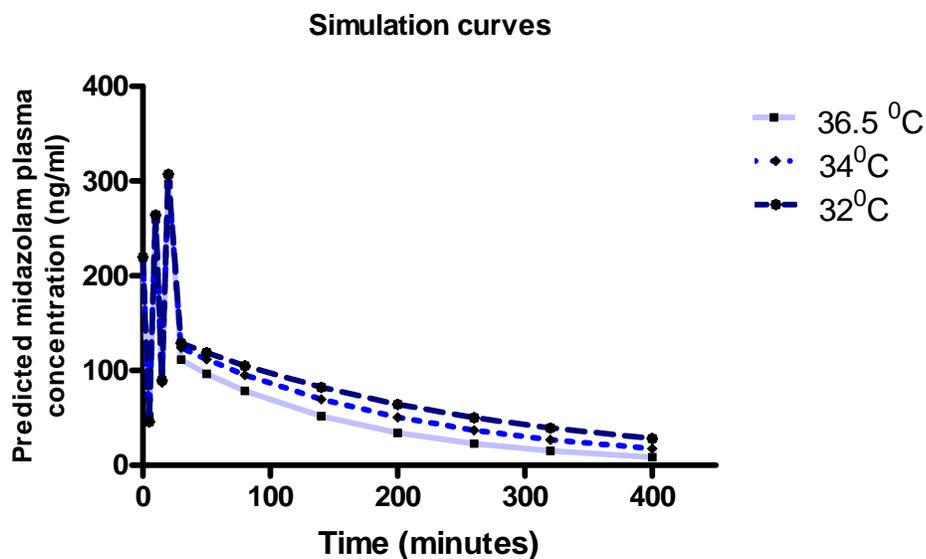


Figure 4-9: Time-concentration simulation curves of midazolam (three dose iv bolus) at 36.5°C (square symbols), 34°C (circle symbols) and 32°C (triangle symbols) using NONMEM estimated parameters. The 32°C profile has the highest AUC and  $C_{max}$  followed by 34°C and 36.5°C curves. The predicted midazolam clearance at 34°C decreased to 74.0% compared to the clearance of core temperature baseline 36.5°C, the predicted midazolam clearance of 32°C decreased to 57.2% compared to the clearance in 36.5°C.

Table 4-2: NONMEM and bootstrap estimates with bootstrap estimated 95% confidence interval

	NONMEM	Bootstrap
	Estimates	Estimates (95% CI)
THETA(1)	18.5	18.4 ( 17.2~19.6)
THETA(2)	9.11	9.65 ( 0.610~18.7)
THETA(3)	230	226 (19.3~433)
THETA(4)	37.3	36.8 ( 28.4~45.2)
THETA(5)	4.24	4.10 ( -4.06~12.3)
THETA(6)	-17.9	-21.3 (-52.2~9.57)
THETA(7)	-5.08	-4.68 ( -8.90~ -0.410)
THETA(8)	-3.75	-4.12 ( -7.01~ -1.20)
ETA(1)	0.0762	0.0618 ( -0.167~0.290)
ETA(2)	0.0543	0.487 (-1.66~2.64)
ETA(3)	0.135	0.100 (-0.199, 0.399)
ERR(1)	0.166	0.169 (0.0605, 0.278)
ERR(2)	0.0992	1.638 (-9.44, 12.7)

$CL = \theta_1 \times (TEM/36.5)^{\theta_5}$ ,  $V_1 = \theta_2$ ,  $TVV_1 = V_1 \times \text{EXP}(\text{ETA}(1))$ ,  $Q = \theta_3 \times (TEM/36.5)^{\theta_6}$ ,  $TVQ = Q \times \text{EXP}(\text{ETA}(2))$ ,  
 $V_2 = \theta_4 + (HR/68)^{\theta_7} + MG \times \theta_8$ ,  $TVV_2 = V_2 \times \text{EXP}(\text{ETA}(3))$ ,  $Y = F + F \times \text{ERR}(1) + \text{ERR}(2)$ .

## 4.5 DISCUSSION

This study demonstrated that midazolam metabolism is reduced by mild hypothermia in normal healthy volunteers. The results provided the basis for predicting alterations in midazolam clearance and possible drug therapy interaction involved of other CYP3A substrates under mild hypothermic conditions. There are two major observations from this study. First, the cold saline 30mL/kg infusion with midazolam and magnesium reduced the core temperature in normal healthy volunteers in the absence of anesthesia, however, the shivering was minimally suppressed in healthy subjects. Second, the clearance of midazolam was significantly affected by body temperature change in human from both non-compartmental pharmacokinetic analysis and population pharmacokinetic modeling. This model predicted that midazolam clearance would decrease 11.1% for each degree lower in core temperature from 36.5°C.

The body temperature changes observed in this study from both normothermia and hypothermia groups were consistent with previous studies. In our normothermia group, warm saline infusion with midazolam resulted in a small  $0.4 \pm 0.2^{\circ}\text{C}$  decrease from baseline. Addition of magnesium resulted in a temperature reduction of  $0.9 \pm 0.3^{\circ}\text{C}$ . Kurz et al. used midazolam and surface cooling to induce hypothermia and showed that midazolam impairs thermoregulatory control by decreasing sweating threshold  $0.3^{\circ}\text{C}$ , and decreasing shivering threshold around  $0.6^{\circ}\text{C}$  (Kurz et al., 2005). Our previous study used just rapid warm saline infusion resulted in  $0.5^{\circ}\text{C}$  temperature reduction (Moore et al., 2008). In our hypothermia group, the lowest core temperature  $35.4^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$  was observed in the cold saline infusion with magnesium group, co-infusion of 4g of magnesium during a cold saline infusion resulted in an additional  $0.5^{\circ}\text{C}$  of cooling compared

with cold saline infusion itself. We have previously reported that rapid infusion of cold saline can effectively reduce body temperature in normal healthy volunteers compared with surface cooling (Moore et al., 2008; Hostler et al., 2009). Magnesium sulfate facilitates cooling through its known vasodilatory effects to promote peripheral heat exchange and blunt the shiver response upon cold saline infusion. Magnesium sulfate in total given dose of 8.75 to 16.75 g has been shown to increase the rate of hypothermia and improve the comfort of healthy subject during hypothermia induction (Zweifler et al., 2004). Previous studies have shown magnesium sulfate significantly reduce the shivering threshold ( $36.3 \pm 0.4$  vs  $36.6 \pm 0.3^\circ \text{C}$ ,  $p=0.04$ ) in healthy volunteers which was consistent with the observation of this study (Wadhwa et al., 2005). In addition, magnesium may provide protection against ischemia in reducing CA1 neuronal death combined with modest hypothermia ( $35^\circ\text{C}$ ) in animal model (Zhu et al., 2005). Therefore, magnesium use in hypothermia deserves further study. Although the gain of temperature changes was small in this study, the infusion was well tolerated in this population and may prove of some benefit in achieving a target temperature in conscious patients. These results suggested that it is possible to reduce body temperature in conscious subjects. However, cold saline infusion with midazolam and magnesium combination used in this study can only create a mild and short duration of hypothermia and did not sufficiently maintain hypothermia to a clinical desired level.

In this study, we evaluated the model compound midazolam as an index of CYP3A4 metabolism. Midazolam is a well-known CYP3A probe mostly because it exclusively metabolized by human CYP3A4 isoforms, and midazolam is not a P-glycoprotein transporter substrate. The primary midazolam metabolite produced through CYP3A in human is 1'-hydroxymidazolam (~70%). Minor metabolites formed by CYP3A4 are 4'-hydroxymidazolam and 1',4'-

dihydroxymidazolam (Galetin et al., 2005). Our results demonstrated that even in mild and short duration of hypothermia, the 1'-hydroxymidazolam formation clearance in the Cold+Mg group was significant lower compared to normothermia. These results were consistent with previous preclinical and clinical studies. CYP3A activity during cooling was previously determined by ethylmorphine-N- demethylation in vitro in piglet liver microsomes. This study demonstrated a temperature dependence with CYP3A activity ( $p < 0.01$ ). Furthermore, this study indicated that the plasma concentration of fentanyl, another CYP3A substrate (liver blood flow dependent), significantly increased during hypothermia ( $31.6 \pm 0.2$  °C) for 6 hours and after rewarming ( $p < 0.001$  and  $p < 0.05$  respectively) (Fritz et al., 2005). The study by Fukuoka et al. reported significant changes of midazolam clearance during moderate ( $32-34^{\circ}\text{C}$ ) hypothermia in eight brain injured patients who were given a continuous infusion of midazolam (Fukuoka et al., 2004). Collectively, previous preclinical or clinical studies have indicated the possible changes of CYP3A activity in cooling, however, the relationship of body temperature and CYP3A activity in human was still not fully elucidated.

Our data analysis comprehensively estimated the pharmacokinetic parameters of midazolam during mild hypothermia in human and predicted the relationship between core temperature and CYP3A activity by using both non-compartmental pharmacokinetic analysis and population based nonlinear mixed effect pharmacokinetic modeling. The non-compartmental pharmacokinetic analysis assumed that the clearance was constant over time within group which may not be a sufficient and sensitive estimate for the changes of systemic clearance with changing temperature. Our study was designed to record core temperature and midazolam concentration continuously over time, therefore, allowing the time varying covariate of

temperature to be modeled using a nonlinear mixed-effects population pharmacokinetic approach. The approach provides for the preservation of data structure so that each individual condition contributes to the population description. The model estimate of clearance of midazolam was 18.5 L/hr at 36.5 °C which was consistent with previous studies (de Wildt et al., 2003; Shimizu et al., 2007; Bolon et al., 2003). The relationship between core body temperature and the midazolam systemic clearance was described using the following function:  $CL (L/hr) = 18.5 \times (TEM/36.5)^{4.24}$ , which provided the basis for predicting the temperature relationship with clearance of midazolam. This population model predicts that a 1°C reduction in core temperature from 36.5°C produces an 11.1% reduction in midazolam clearance. This 11.1% percentage reduction per degree change in core temperature is consistent with the results of Leon et al. who reported that for each 1°C decrease in temperature, a 10% reduction in tissue metabolic requirements and free radical production (Leon 2004). In addition, the study by Caldwell et al. reported that 11.3% clearance of vecuronium decreased with per degree (Celsius) temperature change in healthy human volunteers (Caldwell et al., 2000). A study by Michelsen et al. also demonstrated that the clearance of remifentanyl decreased by 6.37% for each degree (Celsius) below 37°C in patients who undergoing coronary artery bypass surgery (Michelsen et al., 2001). Our pharmacokinetic model predicted a midazolam disposition changed under mild hypothermia condition was consistent with previous studies.

The model suggested a predictive decrease in drug metabolism per degree change in body temperature from simulations at temperature 34°C and 32°C, and the predictive utility of these estimates in a prospective validation cohort remains to be determined. However, this study has

been done in healthy volunteers and there are maybe difficulties to conduct these lower temperature experiments.

Due to the shivering response and discomfort of healthy human subjects, the cooling period of this study was designed for duration of 200 minutes followed by rewarming. Due to the likelihood of alterations in clearance upon rewarming, blood samples were only drawn during the period of time when subjects were actively on the cooling protocol, which was from time zero to 200 minutes. This duration may not be long enough to capture a better midazolam disposition curve, however it was not possible to collect samples at a later time point while accurately estimating the cooling effect on the metabolism of midazolam in the hypothermia healthy human study. In addition, the estimated half-life of midazolam in healthy normothermia control group of this study was consistent with literature as previously mentioned.

In addition, compared to previous animal disease model study results in Chapter 3, in which midazolam clearance has been found to be decreased between hypothermic CA and control group. This effect was believed to be majorly mediated by the CA disease state which is not presented in the current experiment. Hypothermia was associated with reduced the volume of distribution. Future studies to determine specific dosing guidelines for commonly used medications in critically ill patients during cooling are needed to prevent the unrecognized therapy-drug interactions in this highly susceptible patient population. The magnitude of pathway specific alterations in drug elimination continue needed to be elucidated in order to begin to develop for specific dosing recommendations. Until such guidelines are developed, vigilance with drug response monitoring is a clinical necessity.

To further understand the underlying mechanisms of the facts that the metabolism rate decrease with lower body temperature might need to refer to Arrhenius equation:  $K=Ae^{-E_a/KT}$ , where metabolism rate decrease with lower body temperature and it has been explained from the collision theory, transition state theory, and Q10 (temperature coefficient):  $Q_{10}=R_2/R_1^{10/(T_2-T_1)}$  theory.

## 5.0 ASSESSMENT OF FOUR MAJOR DRUG METABOLISM ENZYMES AND RENAL EXCRETION IN HEALTHY HUMAN SUBJECTS VIA THE ADMINISTRATION OF A FIVE-DRUG MICRODOSED COCKTAIL

Zhou J (IRB author and study coordinator), Callaway CW, Hostler D, Rittenberger J and Poloyac SM, *IRB approved, the study is on going*

### 5.1 INTRODUCTION

A central premise that underlies the importance of this thesis research is the overall incidence and importance of adverse drug reactions in critically ill patients. Lazarou and colleagues reported a serious adverse drug reaction incidence rate of 6.7% in US hospitalized patients with 0.32% being fatal (Lazarou et al., 1998). This projected to two million patients and greater than 100,000 deaths annually; making these events between the fourth and sixth leading cause of death. Adverse drug events cause or contribute to 6-7% of hospital admissions and increase length of stay by approximately 2 days (Bates et al., 1997; Classen et al., 1997; Ingelman-Sundberg et al., 2008). Adverse drug events in critically ill patients are particularly problematic because they are more common and often life-threatening (Cullen et al., 1997). The Adverse Drug Event Prevention Study Group found an approximately 2-fold increased risk in intensive care unit (ICU) patients versus non-ICU comparators. In particular, adverse drug reactions with nitrates, opiates, and benzodiazepines are common in ICU patients and are related to increased length of stay (Vargas et al., 1998). Furthermore, prolonged sedation can complicate ventilator weaning, which also increases length of stay and the rate of complications in these patients.

Some adverse drug events, such as propofol infusion syndrome, have fatal consequences in critically ill patients and the potential pharmacokinetic origin of this syndrome remains largely unexplored (Iyer et al., 2009). Studies have stratified adverse drug events based on drug class and determined that significant differences in adverse drug events between ICU and non-ICU patients were observed for cardiovascular, antiarrhythmic, muscle relaxant, and anticoagulant medications. Realizing that combined with the new hypothermia intervention, the understanding for the already complicated pharmacotherapy, disease effects and therapeutic hypothermia on drug metabolism and elimination become an urgent research question.

Although clinical outcome data demonstrates an increased incidence of adverse events, the alterations in drug disposition are understudied due to an inability to assess metabolic changes in the critically ill patient population. The reason for this lack of information is primarily due to an inability to safely assess alterations in drug metabolism in critically ill patients. One potential method presented in this study for phenotypically assessing drug metabolism in critically ill patients is to use very low doses (microdoses) of probe drugs to evaluate drug metabolism. This microdosing strategy typically uses over 10-fold lower than normal pharmacologic doses, thereby, improving the safety profile in critically ill patients and minimizes drug-drug interaction. It is the long term goal of our research to evaluate the utility of microdosing of probe drugs in cardiac arrest patients as a method to determine optimal dosing strategies in these patients, and/or during hypothermia treatment.

In order to further assess the effect of hypothermia on CA patients, the goal of this study is to provide a feasibility experiment to determine if microdoses of probe drugs can be used to

phenotypically assess individual drug metabolizing enzymes as well as renal function in healthy human volunteers. This feasibility experiment will provide essential pharmacokinetic parameter estimates to demonstrate the utility of this approach for subsequent use in patient populations.

Four major enzyme isoforms (CYP1A2, CYP3A4, CYP2C19, and CYP2D6) and renal function will be evaluated. The microdosed probe drugs used in these studies are theophylline (CYP1A2), midazolam (CYP3A4), esomeprazole (CYP2C19), metoprolol (CYP2D6) and famotidine (renal secretion). These drugs were selected based on their use as phenotypic probes availability of an IV formulation for subsequent use in human studies, and based on their biopharmaceutical characteristics (Bates et al., 1997; Frye 2004; Derkenne et al., 2005; Obase et al., 2003; Turpault et al., 2009). The use of an IV formulation is important because CA patients rarely receive oral administration of drugs, therefore, alterations in absorption are an unnecessary confounder to assessment of drug exposure in this population. In addition, esomeprazole, midazolam, and metoprolol were recently validated as a high dose probe cocktail in normal healthy volunteers by Turpault et al (Turpault et al., 2009) and showed no drug-drug interactions.

## 5.2 MULTIPLE PROBE DRUG BIOANALYTICAL ASSAY BY UPLC/MS/MS-FIVE DRUG COCKTAIL

The purpose of this UPLC/MS/MS assay establishment is to provide the analytical tool to ensure the microdosed cocktail probe drug administration feasible due to the sensitivity provided by the UPLC/MS/MS. In addition, the purpose of this assay is also to provide the reproducible quantitative assessment of plasma concentrations of five probe substrates for four different CYP isoforms and renal elimination simultaneously. This simultaneous assessment will allow for the evaluation of these isoform activities from a single study. All probe drugs that are administered as a microdose require a high degree of analytical sensitivity and specificity. Since no previous method has been established in the literature, we set out to establish this assay as an essential step needed for future PK analysis.

### **5.2.1 Probe drugs selection**

As we have discussed in the previous section (see detail in Chapter 2), basic requirements of a probe drug include specificity, no drug-drug interaction, and reasonable half-life. In this cocktail combination, all probe drugs are FDA approved drugs. In addition, all of the drugs in this cocktail are available as an IV formulation. The five probe drugs (theophylline, midazolam, esomeprazole, metoprolol, and famotidine) have been chosen and their metabolism profiles were used to represent the enzyme activities of CYP1A2, CYP3A4, CYP3C19, CYP2D6 and renal secretion in humans. Their specific metabolites (1-hydroxymidazolam, 5-hydroxyomperazole, 1, 3-dimethyluric acid, and 1-hydroxymetoprolol) have also been included in the cocktail method development.

## **5.2.2 Instrumentation and conditions**

As discussed in previous chapters, UPLC/MS/MS assays have been developed. The highest intensity daughter ion fraction with its corresponding collision energy has been used for the selective MS transition for each probe drug. The MS/MS conditions for all analytes have been listed in Table 5-1. Instrument condition establishment is also similar to the previous chapter.

## **5.2.3 Analytical assay development**

### ***5.2.3.1 Calibration standard***

All standard compounds have been ordered from Toronto Chemical Company (Toronto, Canada) and Cerilliant (Round Rock, TX). Blank human plasma (pooled, heparized) was purchased from Central Blood Bank of Pittsburgh (Pittsburgh, PA). Oasis 1cc HLB (hydrophilic-lipophilic balance) and 1cc MAX (mixed mode anion exchanges) solid phase extraction cartridges were purchased from Waters (Milford, MA, USA). All stock solution of probes, metabolites and internal standards are 1.0mg/mL in methanol and stored at -20°C. All glassware used for preparation of calibrations and samples were silanized tubes. Two different reconstitute solutions/further dilution were: A) 80:20 acetonitrile: water for midazolam, metoprolol, esomeprazole and 1-hydroxymidazolam, 5-hydroxyomeprazole; B) 10:90 acetonitrile: water for 1-hydroxymetoprolol, famotidine, theophylline and 1,3-dimethyluric acid.

### ***5.2.3.2 Sample preparation and extraction***

Solid phase extraction (SPE) with HLB and MAX cartridges has been used. HLB column has been used in the previous section for four drug cocktail assay development. MAX column utilize a mixed-mode strong anion exchange and reversed-phase mechanisms to separate both polar and

non-polar, neutral and anionic compounds simultaneously from aqueous media. Typical applications include acidic drugs and their metabolites. In this set of assay development, MAX column has been used to extract theophylline (weak acid) and its metabolite 1,3-dimethyluric acid from plasma and urine samples. The specific procedures for using HLB and MAX extraction have been described in Figure 5-1. SPE choice and reconstitute solution choices refer to Table 5-2.

<b>HLB</b>	<b>MAX</b>
Equilibrate column with 1mL MeOH, 1mL H2O	Equilibrate column with 1mL MeOH, 1mL H2O
Load sample (diluted in water)	Pretreatment sample with 5% NH4OH
Wash with 1mL pure H2O	Load sample on column
Wash with 1mL 5% Formic acid in H2O	Wash with 1mL 5% NH4OH in H2O
Change to clean tube	Change to clean tube
Elute with 2mL pure MeOH	Elute with 2mL 2% FA in MeOH

Figure 5-1: Solid phase extraction procedure for using HLB and MAX cartridges

Table 5-1: SRM transition method for MS/MS (precursor to product) for four parent drugs, four major metabolites and their respective internal standards (IS)

CYP450	Compound	SRM transition	Collision energy (ev)	Polarity	RT (min)	LOD/LOQ (ng/mL)
CYP3A4	Midazolam	325.7>291	27	+ve	3.57	0.05/0.1
	1-Hydroxymidazolam	342>324	25	+ve	3.55	0.05/0.1
	Midazolam-d4 (IS)	330>295	27	+ve	3.57	
CYP2C19	Esomperazole	346>179.9	34	+ve	3.43	0.2/0.5
	5-Hydroxyomperazole	362>151.9	31	+ve	3.57	0.1/0.2
	Omperazole-d3 (IS)	349>135.9	34	+ve	3.43	
CYP2D6	Metoprolol	268>191	18	+ve	3.32	0.2/0.5
	1-Hydroxymetoprolol	284>91	41	+ve	1.46	0.1/0.2
	Metoprolol-d7 (IS)	275.1>191	17	+ve	3.32	
CYP1A2	Theophylline	181>124	23	+ve	1.18	0.5/2.0
	1,3-dimethyluric acid	196.9>169	17	+ve	0.9	0.5/2.0
	Theophylline-d6 (IS)	187>127	23	+ve	1.18	
Renal	Famotidine	338.1>188.9	20	+ve	0.87	0.2/0.5
	Ranidine (IS)	315>175.9	21	+ve	0.99	

Table 5-2: The extraction and reconstitute solution for different compounds

		Reconstitute solution	Extraction method	St curve range
CocktailA	Metoprolol	80:20 Acetonitrile:H2O	SPE/HLB	0.2-500ng/mL
	5-hydroxyomeprazole	80:20 Acetonitrile:H2O	SPE/HLB	0.2-500ng/mL
	esomeprazole	80:20 Acetonitrile:H2O	SPE/HLB	0.2-500ng/mL
	1-hydroxymidazolam	80:20 Acetonitrile:H2O	SPE/HLB	0.2-500ng/mL
	Midazolam	80:20 Acetonitrile:H2O	SPE/HLB	0.2-500ng/mL
CocktailB-1	1-hydroxymetoprolol	10:90 Acetonitrile:H2O	SPE/HLB	1.0-500ng/mL
	Famotidine	10:90 Acetonitrile:H2O	SPE/HLB	1.0-500ng/mL
Cocktail B-2	Theophylline	10:90 Acetonitrile:H2O	SPE/MAX	2.0-500ng/mL
	1,3-dimethyluric acid	10:90 Acetonitrile:H2O	SPE/MAX	2.0-500ng/mL

#### 5.2.4 Method validation

Analytical method validation has been described in aspects of linearity, sensitivity, precision and accuracy, matrix effect and recovery efficiency. A typical chromatography window from UPLC/MS/MS from one of the validation days has been shown in Figure 5-2.

##### 5.2.4.1 Linearity and sensitivity

Every calibration curves consisted of ten concentrations. Calibration curves were constructed using the analytes/IS peak area ratio vs the analyte concentration. Calibration curves were prepared in three individual days with the precision and accuracy testing. Cocktail A analytes (midazolam, metoprolol, esomeprazole and 1-hydroxymidazolam, 5-hydroxyomeprazole) standard curves were ranged from 0.2-500ng/mL. Standard curve range for 1-hydroxymetoprolol and famotidine were 1.0-500ng/mL, and the standard curve range for

theophylline and its metabolites were 2.0-500ng/mL. QC samples are prepared in the low, medium and high range of the curve.

The sensitivity was described from limit of detection (LOD) and limit of quantitation (LOQ). The LOD was determined based on the peak area of lower than 5-digit (when area <10,000). The LOQ was determined based on the peak area at this level can be determined with variability less than  $\pm 20\%$  (accuracy of 80-120%) when repeated five times in extracted samples. The LOQ for eight analytes were between 0.1-2.0ng/mL (0.75-15pg/column). UPLC/MS/MS method significantly decreased the background noise, therefore improved the sensitivity and provided wider range of linearity. Our study and other studies have shown that 500-2500 fold of analysis linearity range are accessible.

#### ***5.2.4.2 Precision and accuracy***

Precision and accuracy of the method were determined by preparing and analyzing QC samples. QC levels at low, medium and high in five replicates were analyzed on the same day to determine the intra-day precision. The process was repeated in three separate days to determine the inter-day precision. Precision was calculated as the coefficient of variation (CV%) of the replicate analysis, whereas accuracy is the percent difference between nominal and observed values (% bias). The acceptable inter-day and intra-day precision and bias were set as <20 % (or accuracy between 80-120%) deviations of nominal concentrations. The intra-day and inter-day precision were < 17.9% for all QCs. The accuracy (%) of all analytes QCs ranged from 92.9-121.6% depends on the analyses. The good precision and accuracy indicate that the analytical methods are reproducible and reliable (Table 5-3).

### 5.2.4.3 Matrix effect and recovery

The recovery efficiency and evaluation of matrix effect were determined by preparing and analyzing QC samples. There are three sets of QC samples for each compound, they are called neat injection, post extraction, and standard extraction (see detail in Chapter 2). The areas of neat samples, post extraction samples and normal extraction samples have been used for calculation of matrix effect and recovery. The matrix effect was calculated as  $(A_{\text{post-extraction}}/A_{\text{DI}} - 1) \times 100$ . Negative value represents the ion suppression effect. The recovery efficiency of every analyte was calculated as  $A_{\text{extraction}}/A_{\text{post-extraction}} \times 100$ . The matrix effect for every analyte was different and ranged from 74.8% to 132.5%. There was a matrix ion suppression effect for most analytes (post extraction response was lower than the neat sample response). For all analytes, the recovery ranged between 74.5-95.7%. Recovery efficiency indicated that the solid phase extraction method was sufficient to recover > 74.5% of compound from standard solution without significant loss in the extraction procedure (Table 5-4).

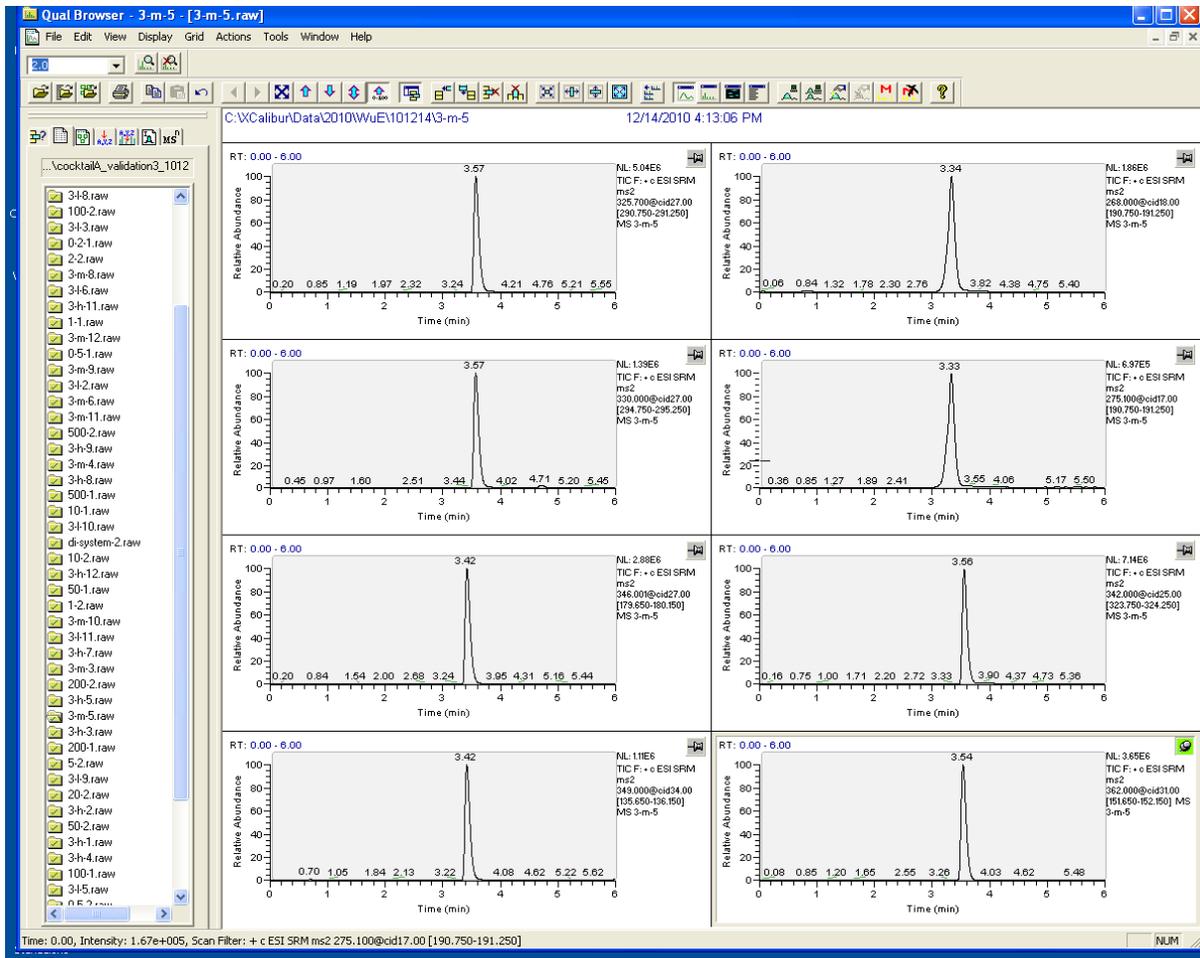


Figure 5-2: Typical chromatography window from UPLC/MS/MS in one of the validation days.

Above showed chromatographies are midazolam, metoprolol, midazolam-d4, metoprolol-d7, esomeprazole, 1-hydroxymidazolam, omeprazole-d3 and 5-hydroxyomperazole (from left to right, then up to down direction)

Table 5-3: Calibration curve ranges, precision and accuracy evaluation of eight analytes. (n=6)

	QC ng/mL	Observed			Accuracy (%)	Precision (CV%)			Standard Curves (three days)
		Day 1	Day 2	Day 3		Day1	Day2	Day3	
MDZ	2.5	2.34±0.10	2.72±0.19	2.45±0.09	98.4±7.92	4.27	7.10	3.62	y1=0.01673+0.09159x R <sup>2</sup> =0.9980
	40	37.9±0.75	42.9±1.38	41.9±1.91	100.4±6.34	1.99	3.12	4.56	y2=-0.01762+0.0997x R <sup>2</sup> =0.9972
	250	256.1±9.59	256.8±7.49	255.7±7.31	102.5±3.29	3.74	2.92	2.86	y3=-0.00347+0.09992x R <sup>2</sup> =0.9981
1-OHMDZ	2.5	2.69±0.15	2.54±0.24	2.44±0.24	103.4±8.74	5.77	9.51	9.76	y1=0.03407+0.11260x R <sup>2</sup> =0.9979
	40	42.3±4.64	40.7±2.86	38.3±5.25	102.1±11.2	10.9	7.04	13.7	y2=-0.01647+0.11848x R <sup>2</sup> =0.9967
	250	266.9±10.6	252.2±15.9	268.1±14.7	105.4±5.68	3.99	6.33	5.47	y3=-0.00496+0.11767x R <sup>2</sup> =0.9934
MET	2.5	2.39±0.43	2.26±0.17	2.53±0.33	95.8±14.3	17.9	7.61	13.0	y1=0.05533+0.07474x R <sup>2</sup> =0.9976
	40	41.2±4.20	44.8±1.76	43.4±1.45	106.4±8.28	10.2	3.93	3.34	y2=0.02858+0.07506x R <sup>2</sup> =0.9991
	250	277.8±18.9	256.8±9.5	248.9±12.2	106.1±7.94	6.80	3.71	4.92	y3=0.03535+0.07079x R <sup>2</sup> =0.9929
1-OHMET	4	3.68±0.67	4.03±0.61	3.47±0.49	92.9±15.5	18.2	15.1	14.2	y1=0.02472+0.03545x R <sup>2</sup> =0.9912
	40	38.8±3.10	40.8±4.96	44.3±3.59	101.3±10.2	7.99	12.2	8.12	y2=0.02651+0.02988x R <sup>2</sup> =0.9854
	250	252.7±35.2	241.3±7.18	268.1±22.4	101.5±11.4	13.9	2.97	8.36	y3=0.06659+0.04817x R <sup>2</sup> =0.9849
ESO	2.5	2.60±0.18	2.60±0.25	2.39±0.08	121.6±17.5	6.84	9.71	3.48	y1=0.02667+0.05885x R <sup>2</sup> =0.9979
	40	43.4±1.41	45.6±1.16	43.5±1.44	110.3±4.27	3.25	2.55	3.32	y2=0.02781+0.05563x R <sup>2</sup> =0.9966
	250	263.2±6.41	264.4±6.23	273.5±16.4	106.4±4.12	2.43	2.35	6.01	y3=0.07000+0.05489x R <sup>2</sup> =0.9969
5-OHOMP	2.5	2.68±0.35	2.39±0.21	2.27±0.14	100.1±13.0	13.1	9.0	6.08	y1=0.03836+0.07918x R <sup>2</sup> =0.9963
	40	45.5±4.63	46.7±1.09	46.4±1.39	115.0±8.58	10.1	2.36	2.99	y2=0.07035+0.08608x R <sup>2</sup> =0.9972
	250	265.1±13.2	286.1±12.1	277.9±22.2	109.4±6.99	4.98	4.23	7.97	y3=0.03535+0.07079x R <sup>2</sup> =0.9929
FAM	4	3.93±0.39	3.42±0.53	3.69±0.45	94.5±19.2	9.91	15.6	12.3	y1=0.04112+0.13508x R <sup>2</sup> =0.9809
	40	33.3±4.36	43.1±2.55	47.3±4.39	96.3±18.4	13.2	5.93	9.27	y2=-0.03508+0.08237x R <sup>2</sup> =0.9091
	250	235.8±31.7	288.9±8.91	298.6±28.7	105.7±15.7	13.5	3.08	9.68	y3=0.013002+0.13404x R <sup>2</sup> =0.9879

Table 5-4: Evaluation of recovery efficiency and matrix effect of probes, metabolites and internal standard (n=6)

Compound	QCs (ng/mL)	Recovery (%)		Matrix effect (%)	
		Value	SD	Value	SD
Midazolam	2.5	80.9	13.6	74.8	11.0
	40	86.6	8.8	76.9	3.9
	250	81.4	16.8	81.8	7.8
1-OHMDZ	2.5	76.5	5.1	108.0	13.6
	40	95.7	3.8	81.6	2.9
	250	74.5	13.8	99.0	8.9
Metoprolol	2.5	94.7	6.4	119.2	15.2
	40	91.1	16.0	108.4	9.7
	250	86.5	9.9	132.5	8.9
1-OHMET	4	94.5	18.7	96.4	13.1
	40	95.2	9.17	98.5	6.2
	250	92.4	3.83	92.3	12.6
Esomeprazole	2.5	86.7	10.3	106.0	12.5
	40	91.6	4.7	94.5	4.9
	250	91.6	15.1	94.6	8.8
5-OHOMP	2.5	86.8	4.2	87.5	10.6
	40	84.9	6.8	83.9	2.7
	250	90.3	14.2	88.1	8.1
Famotidine	4	89.3	15.0	110.1	24.0
	40	101.9	15.8	87.0	12.9
	250	99.1	10.3	79.8	11.1

### **5.2.5 Conclusion**

The cocktail bioanalytical assays for four probe drugs (midazolam, esomeprazole, metoprolol and famotidine) have been established and validated. All assays are selective and reproducible. Total running time is 6.0 minutes per sample. The assays are ready to be used for sample analysis. Theophylline is still on the way to be validated.

Due to the differences in physicochemical characters (e.g. hydrophilic vs hydrophobic, acid vs neutral) of these analytes, this set of cocktail has been divided into two different reconstitute groups and used two different extraction methods for the best sensitivity and selectivity. The dynamic nature of the validation process requires a constant re-evaluation of the method when changes of the research objective or analytical instrumentation.

## 5.3 STUDY PROCEDURE

### 5.3.1 Human subjects

This study was approved by the University of Pittsburgh Institutional Review Board. All race and ethnic groups are eligible for enrollment. Based on our experience in the area of Allegheny County, the actual enrollment of clinical trial has been 50% female subjects and 11% minority subjects.

**5.3.1.1 Inclusion criteria:** Subjects between the ages of 18 and 45 years of age who are not pregnant (for female subject) and who have no known medical problems are eligible for inclusion.

**5.3.1.2 Exclusion criteria:**

- Those individuals who have any known history of current or previous medical problems, including but not limit to: heart disease, history of abnormal heart rhythm 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 disorders, sickle cell disease, anemia or other blood disorders.
- Subjects who are known to have the human immunodeficiency virus (HIV positive) or are otherwise have difficulty fighting infection (weakened immune system).
- Subjects who take any medications (including herbal preparations) with the exception of allergy medications, ibuprofen or Tylenol.

- Subjects who 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, Participating in the study while on these medications may lead to the incorrect estimate of the enzyme activities.
- Individuals with a history of heart disease in family members below the age of forty.
- Individuals who have a known allergy to midazolam or a history of other narcotic allergy.
- Individuals who have a known allergy to metoprolol or a history of  $\beta$ 1-receptor blocker (such as propranolol, atenolol, esmolol) allergy.
- Individuals who have a known allergy to theophylline or other xanthine (such as caffeine, aminophylline, paraxanthine, throbromine) allergy.
- Individuals who have a known allergy to esomeprazole or other proton pump inhibitor (such as Lansoprazole, omeprazole, pantoprazole, dexlansoprazole) allergy.
- Individuals who have a known allergy to famotidine or other H<sub>2</sub>-receptor antagonist (such as cimetidine, ranitidine, and nizatidine) allergy.
- Subjects who have a history of long term benzodiazepine use.
- Individuals who are currently ill with a viral or other infection.
- Individuals who have kidney problems or renal insufficiency.
- Individuals who 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.
- Individuals who currently smoke or use any other recreational drugs.
- Individuals who are morbidly obese (very overweight-Body Mass Index>36).

— A body weight of less than 80 pounds. Any subject who met any of these criteria would be excluded from the study.

### ***5.3.1.3 Recruitment procedures***

An announcement was made in Center for Emergency Medicine, Department of Emergency Medicine, and School of pharmacy courses and grand rounds. Recruitment flyers were placed in common areas of the University. Volunteers were given a phone number to call to arrange for an appointment with an investigator. Informed consent and screening took place at that meeting. Subjects are 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.

## **5.3.2 Drug dosage and IV formulation**

### ***5.3.2.1 Microdose simulation***

One of the challenges of this study is to define the appropriate “microdosage”. First, all probe drugs need to be at doses at 1/100th - 1/10th (one to the hundredth to one to the tenth fold) of the doses typically used for treating disease. In addition, the cocktail analytical assays established in the laboratory must provide a lowest limit of detection that allows for the assessment of concentrations throughout the time-concentration profile. Therefore, the complete time concentration profile can be accurately quantified. Thirdly, the study duration has to cover on 3-5 half-life of all probe drugs. Fourth, we used literature reported clearance (CL) and volume of distribution (V) to simulate the elimination and time concentration profile.

Combined with above mentioned total study duration, analytical limit of detection, clinical dose, available PK parameters, this “microdosage” have been determined by performing PK simulation using simple one compartment model (Figure 5-3). The lowest doses that produce the detectable concentration for the entire sampling time have been chosen for this study for each probe drug. The final dosage chosen for these probe drugs are theophylline 1.0mg, midazolam 0.1mg, esomeprazole 0.2 mg, metoprolol 0.5mg and famotidine 1.0mg. Detailed information refers to and Table 5-5.

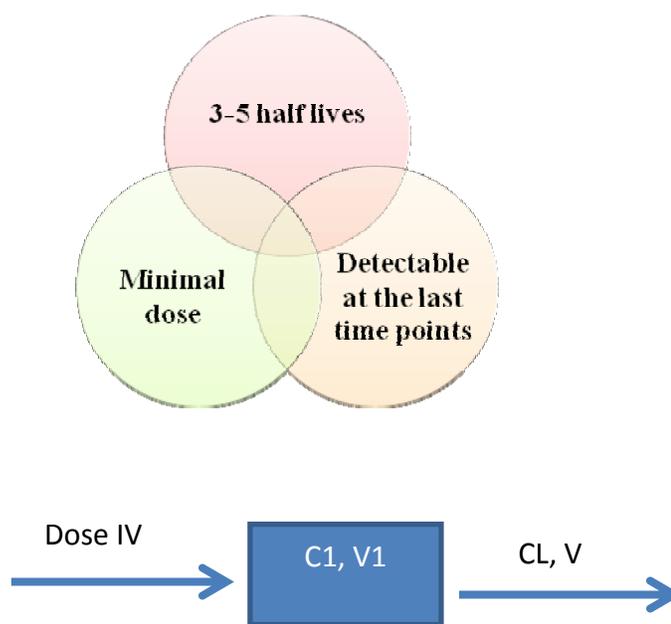


Figure 5-3: Microdose simulation rationale.

### 5.3.2.2 Microdosage and formulation

Microdosage cocktail formulation (theophylline 1.0mg, midazolam 0.1mg, esomeprazole 0.2 mg, metoprolol 0.5mg and famotidine 1.0mg) has been prepared by Investigational Drug Services of

University of Pittsburgh Medical Center. The IV formulation are made of: 1) one aminophylline 1.25mg/2.5mL syringe (note that 1.25 mg of Aminophylline is equivalent to 1 mg of Theophylline), 2) one esomeprazole 1mg/2ml syringe, 3) one 6mL syringe containing three medications: famotidine 1mg/2mL, metoprolol 0.5mg/2mL, midazolam 0.1mg/2mL. These IV doses have been given to subjects at approximately the same time at the PK time zero.

Table 5-5: Typical IV pharmacological dose and the proposed microdosages for five probe drugs

Isoform	Probe drugs	Typical IV Clinical Dose	Human Micro-Dose	Dose proposed for the study	Half Life	Sample Time	Expected Conc.	UPLC/MS/MS requirement
CYP1A2	Theophylline	0.4 mg/kg/hr (400 mg)	0.112 mg (1.6µg/kg)	1mg (<1/100th)	8.7 (6.1-12.8) hours	24 hours	0.486ng/mL	0.2ng/mL
CYP3A4	Midazolam	1.0-4.0mg	0.1mg (1.43µg/kg)	0.1mg (<1/10th)	3 (1.8-6.4) hours	12 hours	0.0687ng/mL	0.2ng/mL
CYP2C19	Esomeprazole	20-40 mg	0.07mg (1.0µg/kg)	1mg (<1/20th)	1-2 hours	8 hours	0.109ng/mL	0.4ng/mL
CYP2D6	Metoprolol	0.2mg/kg	0.056mg (0.8µg/kg)	0.5mg (<1/20th)	4 (3-7) hours	20 hours	0.0098ng/mL	0.02ng/mL
Renal Secretion	Famotidine	10-20mg	0.5mg (7.14µg/kg)	0.5mg (<1/20th)	2.5-3.5 hours	16 hours	0.1365 ng/mL	0.5ng/mL

### 5.3.3 Study procedure

#### 5.3.3.1 Screening

A study physical performed a history and physical exam on each subject to assess for the presence of medical conditions. The screening took place in the department of emergency medicine offices on a visit separate from the experiment protocol. Body composition of the subject was assessed by measuring skinfolds at three sites. Blood samples were collected for sodium, potassium, TSH, SGOT, SGPT, alkaline phosphatase, bilirubin, blood urea nitrogen, creatinine and a complete blood count to exclude undiagnosed liver, hematologic and metabolic

disease. Two tubes of blood have been collected (one gold top and one lavender top tube) for each subject. Normal lab values used by the University of Pittsburgh Medical Center Presbyterian Hospital lab have been accepted as the normal range. In the event a lab value is outside the normal range, but not clinically significant in the study physician's opinion, the subjects will be allowed to repeat the lab test once. Subject had two lab tests outside the normal range will be excluded. Subject excluded from the study has been provided the information concerning their exclusion and they were referred to their primary care physician.

### ***5.3.3.2 Experiment protocol***

Prior to the protocol, subjects have been asked to refrain from caffeine, alcohol and grapefruit juice from 48 hours. Grapefruit juice is known to alter cytochrome P450 metabolism and caffeine and alcohol may alter hydration status. There is no randomization or control group since all the subjects will receive the same treatment. To ensure safety, a defibrillator, airway management supplies, ACLS medications, and reversal agents are available during the protocol. A study physician has been presented for the entire protocol. Upon arriving for a protocol on the first day, subjects will void their bladder and be weighted. All females will undergo a urine pregnancy test at this time. Subjects then have been prepared as follows:

#### ***5.3.3.2.1 Intravenous access and blood sampling***

An 18-gauge intravenous catheter was placed in one of the subject's hand, forearm, or antecubital veins using standard technique. Subjects received IV bolus of cocktail drugs. After the IV bolus injection, 10 mL saline was used to flush the line. Blood samples later were drawn from the same catheter. Blood samples (3.0mL) were collected at zero, 0.25, 0.5, 1, 2, 4, 6, 8, 10,

12 and 24 hours for a total of 33mL of blood taken. Since the total sampling time is 24 hours, the protocol started from the first day and was completed early the next day. Blood samples from 0-12 hours has been collected on the first day, blood sample at 24 hour time point has been collected on the second day visit. Blood was separated by a microcentrifugation and the plasma was stored at -80°C until further analysis. Timeline of the protocol has been described in Figure 5-3.

#### *5.3.3.2.2 Vital signs*

Heart rate and blood pressure have been measured at the subject arrival on the first visit, immediately before the drug administration, 5 minutes and 10 minutes after drug administration and at each blood draw time point afterwards.

#### *5.3.3.2.3 Urine collection*

Total urine has been collected throughout the study, the container has been provided to the subjects for the overnight. The urine concentration of each specific metabolite provides important information of formation clearance of each probe drugs. The formation clearance is calculated from the concentration of the metabolite in the urine times total urine volume divided by the area under time plasma concentration curve.

#### ***5.3.3.3 Patient safety and protocol determination***

Primary endpoints for this protocol are completed after the 24 hours blood sampling and urine collection or 2) if the subject request termination. The following criteria will results in early termination of the study as well: 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) any sign of arrhythmia. All subjects will be monitored for a minimum of 30 minute after completing the study next day. Subjects must void their bladder and be weighted prior to discharge.

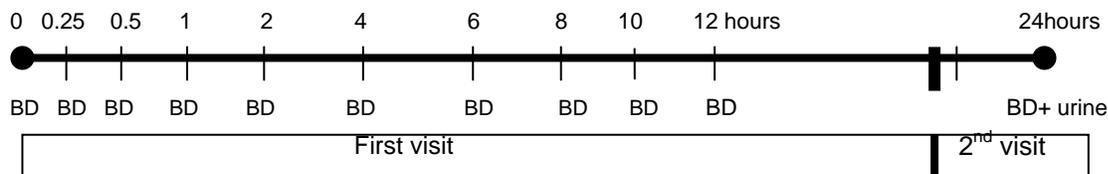


Figure 5-4: Timeline of the protocol (BD= blood draw). Numbers indicate hours.

### 5.3.4 Sample Analysis

Plasma concentrations of midazolam, esomeprazole, metoprolol and famotidine has been determined using UPLC/MS/MS methods described above. Briefly, a 0.20mL aliquot of plasma sample with four specific duterated internal standard (midazolam-d4, omprazole-d3, metoprolol-d7, and ranitidine) was extracted from solid phase extraction column using HLB cartridges. Standard curve and/or QC samples were prepared and run in the same sequence.

## 5.4 PK DATA ANALYSIS

The primary statistical analysis has been based on best describing the complete time concentration curves of the each individual and their pharmacokinetics estimates. Non-compartmental pharmacokinetic analysis has been used for estimating all probe drugs. Pharmacokinetic parameters (CLs, Vd, T1/2, etc) will be compared with literature reported values when these drugs are used in higher concentration in human study.

### **5.4.1 Time-concentration curves**

Time concentration curves of midazolam 0-12 hours, esomeprazole 0-4 hrs, metoprolol 0-12 hours and famotidine 0-12 hours has been showed in Figure 5-5. All sample concentrations in this duration can be accurately measured by previous established analytical assays.

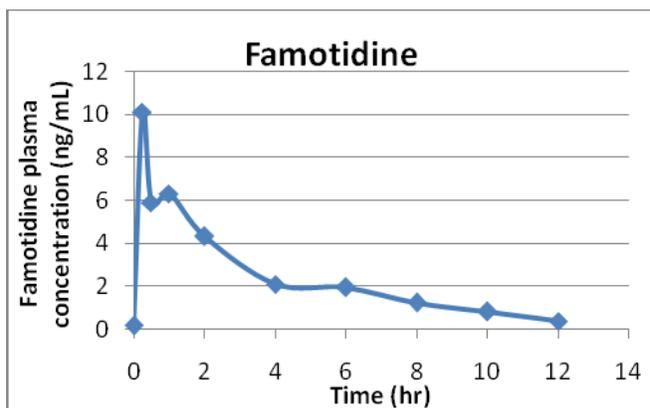
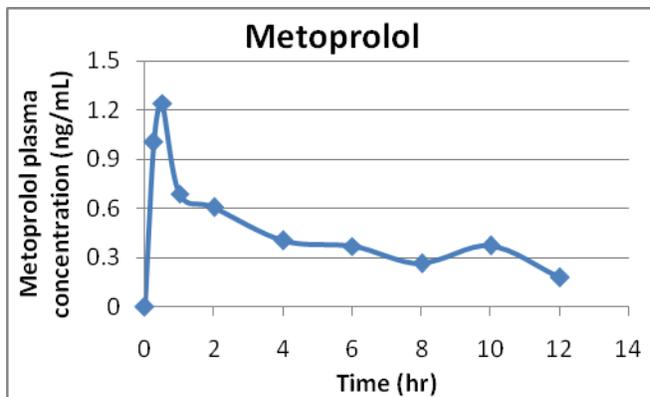
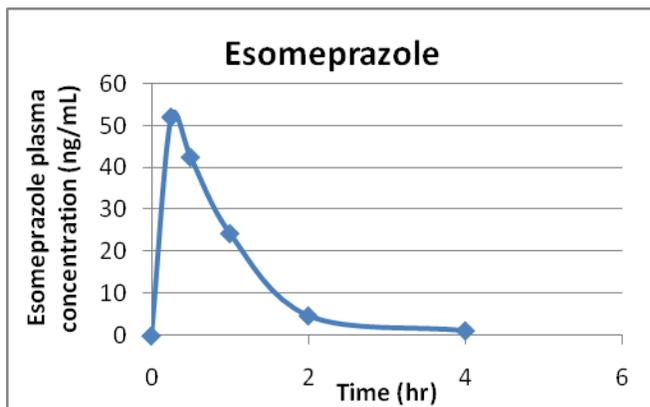
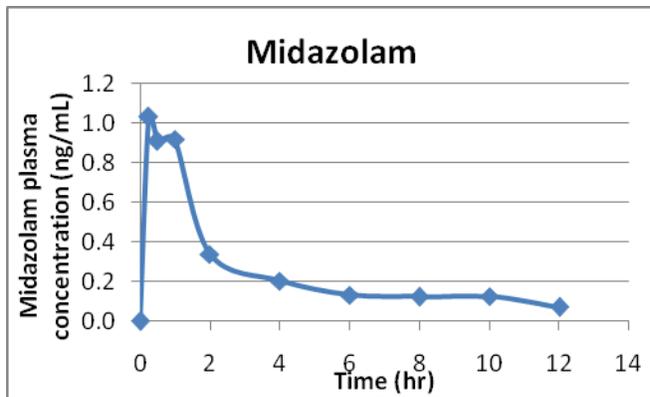


Figure 5-5: Time concentration profile of midazolam, esomeprazole, metoprolol and famotidine of Subject 1.

### 5.4.2 Non-compartmental analysis

Non-compartmental model analysis has been performed by Winnonlin 5.2. PK parameter estimates of  $K_e$ ,  $T_{1/2}$ ,  $T_{max}$ ,  $C_{max}$ ,  $AUC_{0-last}$ ,  $AUC_{0-inf}$ ,  $V_z$  and  $CL$  of each probe drug have been listed in Table 5-6. All  $AUC_{0-last}/AUC_{0-inf}$  ratio is great than 90%, indicates our sample time is sufficient to describe the complete elimination profile and provide accurate estimates from IV dose administration. Compared to multiple previous literature, there is no significant difference from PK estimates of subject 1 with previous literature reported PK estimates (Table 5-7). This indicates the linear PK across the dose range from normal dose to microdose used in this study for midazolam, esomeprazole, metoprolol and famotidine.

Table 5-6: Non-compartment model analysis for midazolam, esomeprazole, metoprolol and famotidine of subject 1.

	<b>Midazolam</b>	<b>Esomeprazole</b>	<b>Metoprolol</b>	<b>Famotidine</b>
$K_e$ (1/hr)	0.3528	0.9577	0.1901	0.2103
$T_{1/2}$ (hr)	1.965	0.7238	3.646	3.297
$T_{max}$ (hr)	0.25	0.25	0.5	0.25
$C_{max}$ (ng/mL)	1.035	51.92	1.244	10.11
$AUC_{last}$ (hr×ng/mL)	3.046	55.40	5.155	28.55
$AUC_{INF}$ (hr×ng/mL)	3.248	56.66	6.093	30.34
$V_z$ (mL/kg)	1023.2	216.09	5068.07	920.40
$Cl$ (ml/hr/kg)	360.9	206.9	963.6	193.5

Table 5-7: Pharmacokinetics parameters of midazolam, esomeprazole, metoprolol and famotidine estimated from our study and from previous literature.

(Mean ±SD)	Author (Reference)	IV Dose (mg)	CLs (ml/min/kg)	Vss/Vd (L/kg)	T1/2 (hrs)
Midazolam	THIS STUDY	0.1	6.02	1.02	1.96
	(Persson 1987)	NA	6.9	1.94	3.1
	(Patel 1990)	3.75	7.87	NA	3.0
	(Allonen 1981)	0.075mg/kg	7.17±1.5	NA	2.4±0.8
	(Greenblatt 1984)	2.5-5.0	7.8-9.4	1.3-2.0	2.1-2.6
	(Greenblatt 1981)	12.5	8.1±0.52	1.72±0.05	2.5±0.2
	(Smith 1981)	5.0	6.38	NA	1.77±0.83
	(MacGilchrist 1986)	NA	10.4±1.3	1.15±0.15	1.6±0.3
	(Thummel 1994)	NA	6.5	0.78±0.34	2.55±2.0
	(Malacrida 1992)	6-15mg/hr	4.9	0.9	2.3
	(Majumdar 2007)	2.0	6.39±0.72	NA	3.7
	(Hostler 2010)	6.0	4.49±0.56	0.779±0.167	1.78±0.347
	(Rogers 2002)	2.09±0.3	8.1±1.31	NA	2.03±0.32
	(Heizmann 1983)	0.15mg/kg	4.61±1.23	0.717±0.16	2.29±0.42
Metoprolol	THIS STUDY	0.5	16.1	5.07	3.65
	(Jordo 1980)	20	11.4±1.4	NA	4.1±1.0
	(Kendall 1982)	NA	NA	NA	3.3±0.7
	(Regardh 1981)	20	11.6±1.6	NA	4.2±1.1
	(Kelly 1985)	50	22.2±1.1	4.7±0.3	2.5±0.3
Eso/ omeprazole	THIS STUDY	1.0	3.45	0.216	0.72
	(Naesdal 1986)	20mg	8.03±0.89	V1=0.173; V2=0.374	0.57±0.059
	(Landahl 1992)	20mg	7.14	NA	0.7-1.0
	(Jacqz-Aigrain 1994)	NA	3.83	0.45	0.86
Famotidine	THIS STUDY	0.5	3.23	0.92	3.3
	(Inotsume 1989)	20 mg/oral	3.86±0.33	NA	3.0±0.5 (SE)
	(Lin 1988)	NA	4.43	NA	NA
	(Smith 1985)	NA	NA	NA	3.8
	(Kroemer 1987)	20mg	4.41	1.13	4.0
	(Takabatake 1985)	20mg	5.89±1.79	Vss=1.14 V1=0.34±0.1	2.59±0.73

NA: Data not available. To match the same unit, certain units from original literature have been converted (such as hour to minutes, ml/min to ml/min/kg).

## 5.5 DISCUSSION AND FUTURE DIRECTION

This feasibility study demonstrated in our first subject that our proposed 1) microdosage, 2) IV formulation, 3) study duration, and 4) UPLC/MS/MS analytical detection limits allow for the determination of PK parameter estimates via multiple microdosed probe drugs. Compared to multiple previous literature, our subject 1 data provide the preliminary evidence that all PK parameter estimated in a microdosing strategy is similar with high dose PK study. This study is descriptive in nature and will be used to determine feasibility, refine protocol and power a full trial of pharmacokinetic study larger sample size and in further patients' population.

Probe drug midazolam, esomeprazole and metoprolol are chosen for specific CYP3A4, CYP2C19 and CYP2D6 activity in human. The metabolism of these probe drugs will be highly dependent on the specific isoform activities. Multiple previously discussed studies have indicated altered metabolism or volume of distribution of midazolam in critical ill patients. Omeprazole metabolism is strongly correlated with CYP2C19 polymorphism. The total clearance can range from  $1369 \pm 516$  ml/hr/kg to  $89.5 \pm 15.4$  ml/hr/kg and T<sub>1/2</sub> ranging from  $0.5 \pm 0.08$  hour to  $2.1 \pm 0.08$  hours, based on the specific genotype patterns (Ieiri et al., 1996). Metoprolol is a commonly used drug in critically ill patients, the microdosing approach will allow for both phenotypic assessment and also informative PK information for this common clinically administered medication.

Famotidine is mainly excreted unchanged by the kidney. Therefore it is a probe drug for renal function. The renal clearance of famotidine exceeded the creatinine clearance, suggesting the

secretion is the major route of elimination of famotidine. The impairment of renal function may lead high blood concentrations of famotidine (Takabatake 1985). Total clearance of famotidine is strongly correlated with creatinine clearance. Previous study has suggested that the renal clearance of famotidine would decrease in direct proportion to the reduction in CL<sub>cr</sub>, which can be calculated as:  $CL=0.2370CL_{cr}+3.193$  (Takabatake 1985). Hypothermia effect on renal function is largely unknown, and is one of our future research questions.

These microdoses at midazolam 0.1mg, esomeprazole 1.0mg, metoprolol 0.5mg and famotidine 0.5mg can potential provide multiple advantages over other normal dose PK design. It will largely reduce the possible drug-drug interaction among the probe drugs and the drug-drug interaction between probe drugs and other medications. This study will prove the first important concept that the current microdosed design and cocktail IV approach can be used for phenotyping human CYP isoform CYP1A2, CYP3A4, CYP2C19 and CYP2D6 as well as renal secretion. This study is an important preliminary data for resubmitting the new clinical studies to evaluate the effect of therapeutic hypothermia on drug metabolism in cardiac arrest patients.

In addition, a high dose probe cocktail study use the same experiment design may needed in the future to confirm the linearity PK across dose range, no drug-drug interaction and provide the validation with other studies or with the regular doses.

## 6.0 THE EFFECT OF THERAPEUTIC HYPOTHERMIA ON PHENYTOIN POPULATION PHARMACOKINETICS IN PEDIATRIC TRAUMATIC BRAIN INJURY PATIENTS

Jiangquan Zhou (Population PK modeling data analysis)

### 6.1 INTRODUCTION

As an extension of our previous research with CYP probe drugs, we sought to evaluate the effect of therapeutic hypothermia on the disposition of clinically relevant drugs in susceptible patient populations. One such patient population includes neonates undergoing therapeutic hypothermia following traumatic brain injury. Frequently, these patients require the prophylactic administration of anti-convulsant medications after insult. Phenytoin is one of the most commonly used anti-convulsant drugs in critical care medicine. In addition, phenytoin is a drug with a narrow therapeutic index, which exhibits non-linear pharmacokinetics. Therefore, this study is to evaluate the effect of hypothermia on phenytoin population pharmacokinetics in pediatric patients. In this study, we have focused on applying population PK modeling and simulation approach to determine the population PK characteristics of phenytoin and the correlation between temperature and phenytoin PK parameters in pediatric TBI patients.

Quite often, we observed the hypotension in rewarming patients after hypothermia treatment and especially in pediatric patients. A previous study has shown that hypothermia significantly decreased the clearance of phenytoin in traumatic brain injury patients with no change in protein binding during hypothermia (Iida et al., 2001). Decreased clearance maybe directly related to its

side effect, for example hypotension, therefore, further clinical trial and data analysis incorporate temperature as covariate to estimate the effect of hypothermia on phenytoin PK is necessary for dose regimen design and response prediction.

### **6.1.1 Phenytoin and its PK characteristics**

Phenytoin (5,5-diphenylimidazolidine-2,4-dione) is a commonly used anticonvulsant in critical care medicine. It is used to control certain type of seizures, and to treat and prevent seizures that may begin during or after surgery to the brain or nervous system. Phenytoin acts to suppress the abnormal brain activity seen in seizure by reducing electrical conductance among brain cells by stabilizing the inactive state of voltage-gated sodium channels (Ya et al., 1986; Talwar 1990; White 1999; Eriksson et al., 2009). Phenytoin is also an anti-arrhythmic and a muscle relaxant (Carson et al., 1979; Somberg 1985; Bebin et al., 1994).

Phenytoin is primarily metabolized by CYP2C9 and CYP2C19. In addition, phenytoin pharmacokinetics follows nonlinear elimination characteristics and saturable metabolism, an increase in the dose would result in a more than proportionate increase in the drug AUC, because the clearance of the drug decreases with an increase in the dose. Furthermore, phenytoin is a high protein binding drug (>90% bound to plasma albumin). Potential protein binding change could also alter the plasma concentration and AUC significantly.

### **6.1.2 Michaelis-Menten kinetics**

When enzyme-catalyzed reactions are saturable, their rate of catalysis will not show a linear response to increasing substrate. The Michaelis-Menten kinetics model is the most commonly

used nonlinear model, which can be described as :  $V=V_{max} \times [S]/(K_M+[S])$ . If the initial rate of the reaction is measured over a range of substrate concentrations ( $[S]$ ), the reaction rate ( $v$ ) increases as  $[S]$  increases. As  $[S]$  gets higher, the enzyme becomes saturated with substrate and the rate reaches  $V_{max}$ , the enzyme's maximum rate. Michaelis constant ( $K_M$ ) is the substrate concentration at which the reaction rate reaches its half-maximum value ( $V_{max}/2$ ). The maximum rate of metabolism ( $V_{max}$ ) is dependent on the amount (or concentration) of enzymes available for metabolism of the drug, while  $K_M$  is the concentration which produces half of  $V_{max}$  and is inversely related to the affinity of the drug to the enzyme (the higher the affinity, the lower is  $K_M$ ).

Under certain circumstances (disease states, age, and drug interactions), the  $V_{max}$  and  $K_M$  values of a drug may change (Mehvar 2001). For example, concurrent administration of carbamazepine or phenobarbital with phenytoin results in an increase in the  $V_{max}$  of phenytoin (enzyme induction). Therefore, it may be necessary to increase the dosing rate of phenytoin if phenobarbital or carbamazepine is added to a patient's regimen. Conversely, hepatic cirrhosis causes a decrease in the  $V_{max}$  of phenytoin, resulting in higher plasma concentrations if the same dose is administered. Additionally, drugs such as cimetidine or chloramphenicol cause an increase in the  $K_M$  value of phenytoin, resulting in higher than expected plasma concentrations of phenytoin (Tozer et al. 1992).

The goal of this study is to define the effect of therapeutic hypothermia on metabolism of this highly clinical relevant drug, phenytoin, and its nonlinear PK change from clinical data set.

Therefore, to obtain a better understanding and prediction for phenytoin time-concentration, response and dose regimen design during hypothermia for clinics.

## 6.2 DATA FOR PK ANALYSIS

### 6.2.1 Subjects and dose information

A total of 19 pediatric patients are included. Ten received hypothermia treatment and nine of them were normothermia control. Subject weights are within the range of  $43.3 \pm 22.4$  kg. All patients received a loading dose  $15.4 \pm 2.96$  mg/kg. The actual maintenance dosages ranged between 1.82-10 mg/kg were given to patients for every 12 hours until the last given dose time at  $194.6 \pm 38.7$  hrs based on clinical need and hospital pharmacodynamics monitoring.

### 6.2.2 Temperature

Time-temperature profiles of all subjects have been described in Figure 6-1. Normothermia temperature has been maintained at  $37.1 \pm 0.6$  °C (Mean $\pm$ SD). Hypothermia has been maintained at 32-33 °C for 48 hours, followed by a slow rewarming at the rate of 1 degree every 12-24 hours.

### 6.2.3 Time-concentration profile

All time-concentration (total) profiles have been shown in Figure 6-2, also with phenytoin dose regiment has been shown in Figure 6-3.

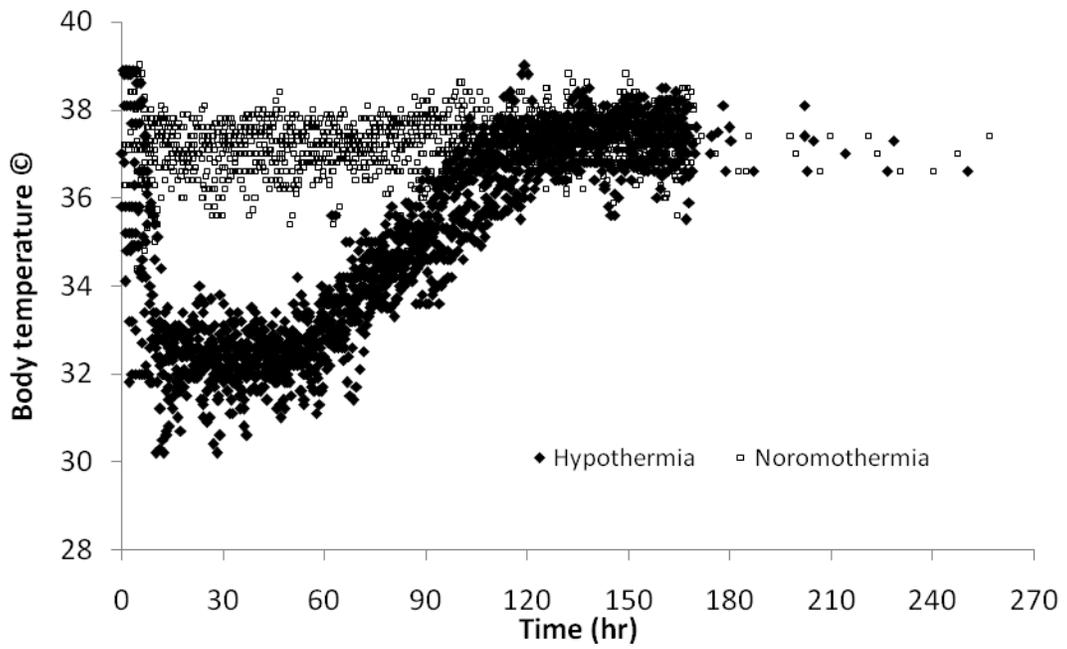


Figure 6-1: Body temperature in hypothermia and normothermia

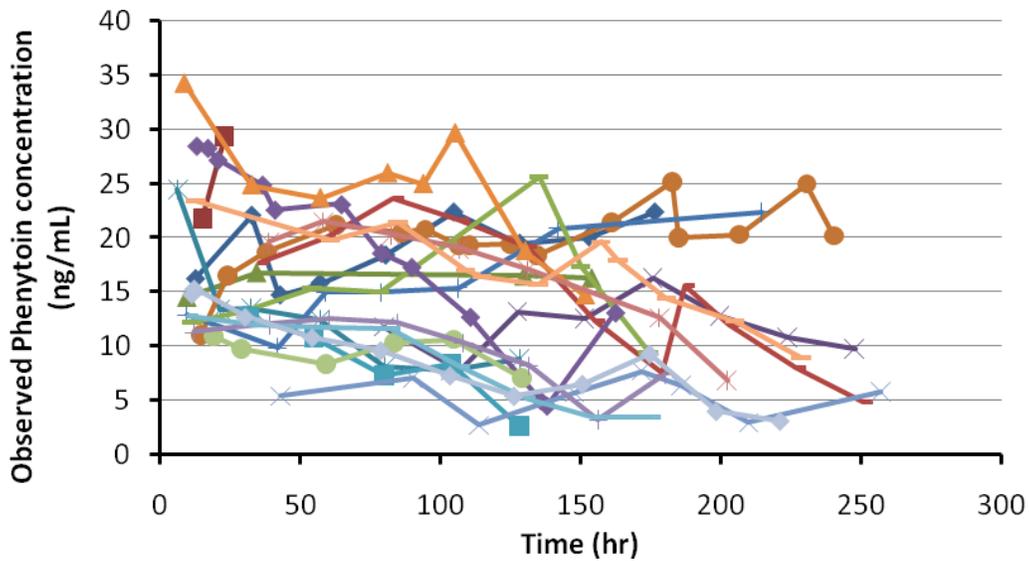


Figure 6-2: Phenytoin time-concentration (total) of pediatric TBI patients

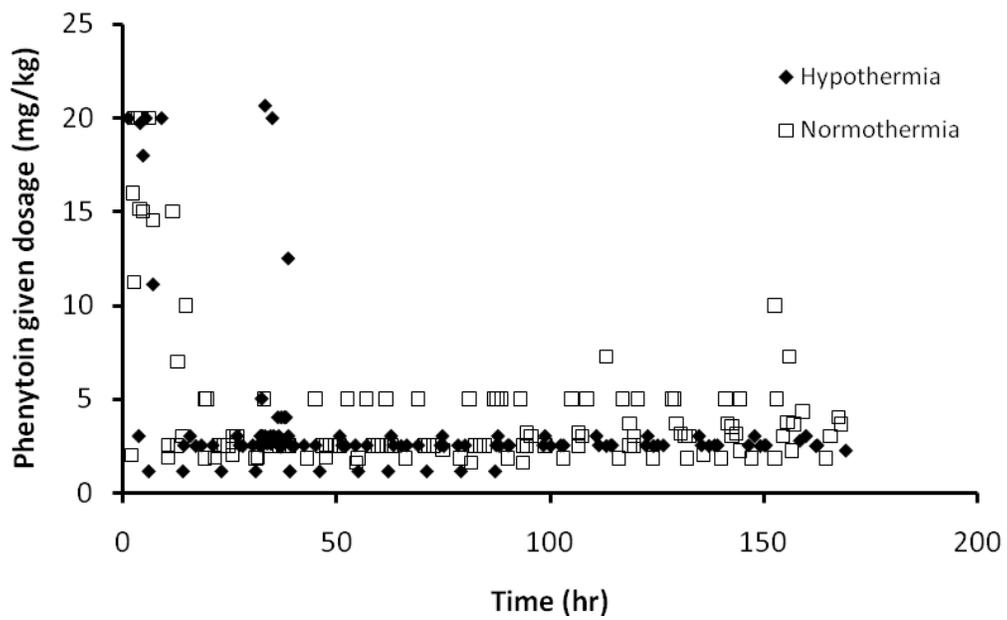


Figure 6-3: Phenytoin dose regiment for hypothermia and normothermia patients

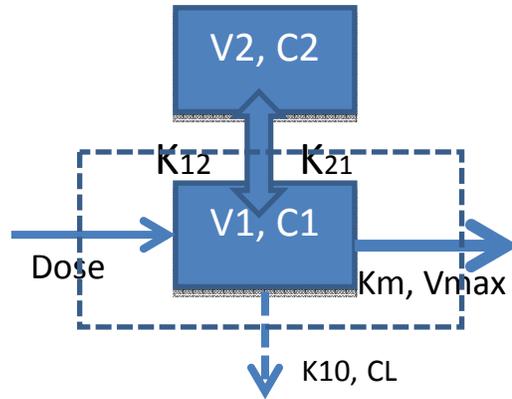
### 6.3 POPULATION PK ANALYSIS

### 6.3.1 Base model

The population pharmacokinetic model was constructed using a nonlinear mixed-effects approach as implemented in the NONMEM VI program (Icon, Hanover, Maryland). The pharmacokinetics of phenytoin generally is believed to follow the nonlinear characteristics. For this set of data, I tested four different structure models looking for the best fitting, and they are:

- 1) one compartment first order elimination (CL, V)
- 2) two compartment model first order elimination (CL, Q, V1, V2 )
- 3) one compartment model nonlinear elimination (Km, Vmax, Vd)
- 4) one compartment model linear parallels with nonlinear elimination ( Km, Vm, CL,V)

Model building process details is the same as previous section. Inter-individual variability in model parameters will be modeled using an exponential term.  $\theta_i = \theta \times e^{\eta}$ . Residual variability, was evaluated using additive, proportional or a combined additive and proportional model  $C_{obs,ij} = C_{pred,ij} \times (1 + \epsilon_{ij1} + \epsilon_{ij2})$ , Where  $C_{obs,ij}$  is the  $j$ th measured observation in individual  $i$ ,  $C_{pred,ij}$  is the  $j$ th model predicted value in individual  $i$ ,  $\epsilon_{ij}$  is the residual error. Further refinement of the error model was performed if needed. The model building process and evaluation are the same as previous section. NONMEM subroutine ADVAN 10 was used is the building nonlinear function although the subroutine ADVAN 6, 9, and 13 can also be used when providing the nonlinear equations in the control file (Figure 6-4).



one compartment model linear elimination:

$$C = C_0 * e^{-kt}$$

$$DCDT(1) = -K_{10} * C(1)$$

$$CL = K_{10} * V_1$$

two compartment linear elimination model:

$$DCDT(1) = -K_{10} * C - K_{12} * C(1) + K_{21} * C(2)$$

$$DCDT(2) = K_{12} * C(1) - K_{21} * C(2)$$

**one compartment nonlinear model :**

$$DCDT(1) = -C_1 * VM / (KM + C_1)$$

one compartment with linear parallels with nonlinear:

$$DCDT(1) = -C_1 * VM / (KM + C_1) - K_{10} * C(1)$$

Figure 6-4: One or two compartment model with nonlinear or first order linear structure for phenytoin base model

### **6.3.2 Covariate model and selection**

The covariate model analysis was constructed by estimating temperature (TEM/37) as a continuous covariate on all PK parameters. To assess whether the model with covariate statistically improved the fit to the data from base model, the difference between their objective function values, referred to as the log likelihood ratio, was calculated (detail also refer to Chapter 3). This ratio was assumed to be chi-square distributed, which means that a reduction in objection function of 3.84 is considered to be significant ( $p < 0.05$ , 1d.f.).

### **6.3.3 Model evaluation**

The final covariate model was evaluated using diagnostic plots, successful minimization, precision for all parameters (se%) and a nonparametric bootstrap. The nonparametric bootstrap approach using sampling with replacement was used to assess the robustness of the model estimates (Parke et al., 1999). Sampling with replacement involved creating a series of datasets of equal size to original data set that are generated by repeatedly sampling individuals from the original dataset, removing these individuals and replacing them at random. The model was refitted to each new data set and this process was repeated 100 times. The stability of the final model was evaluated by examining the bootstrap median and percentiles of model parameter estimates from bootstrap. The Wings for NONMEM (G77 Fortran with WFN 408b) implementation for bootstrapping was utilized.

### **6.3.4 Simulation**

Based on the final model estimates, simulations have been performed comparing three groups in normothermia, hypothermia with 96 hours rewarming and hypothermia with 48 hours

rewarming. Temperature covariate used in the simulation follows the original protocol, which has been described in three phase (induction, maintenance and rewarm). Dosage used for simulation is 20mg/kg for loading dose, with 2.5mg/kg every 12 hours maintaining dose. The total simulation duration is from 0-200 hours.

## 6.4 POPULATION PK MODEL RESULTS

### 6.4.1 Base model selection

Four base model structures have been tested. The OFV values and parameter estimations have been shown in Table 6-1. We decided to pursue model 3 as it provided the best OFV value for estimating three parameters. In addition, this one compartment nonlinear model matches majority of previous literature. Therefore, this one compartmental nonlinear elimination model was chosen for base model structure for phenytoin. Three parameters  $V_{max}$ ,  $K_m$  and  $V_d$  have been estimated in this model using ADVAN 10 subroutine in NONMEM. Two inter-individual variability ( $\omega^2$ ) were chosen for  $K_m$  and  $V_d$ . Residual error model used was  $C_{obs\ ij} = C_{pred\ ij} + Err1$ . This base model provided an adequate description of the data, as judged by visual inspection of diagnostic plots as well as the standard error estimates for each parameter. Using one compartment nonlinear base model, we have estimated the PK parameters for normothermia, hypothermia patients separately and then combined data set (Table 6-2).

Table 6-1: All Base model estimates

Model 1	OFV	Estimates (SE%)
CL, V, 2etas Y=F+ERR(1)	580.127	CL=0.0152 L/hr/Kg (0.231%), V=1.13 (8.42%), ETA1=0.433 (16.2%), ETA2=0.718 (3.27%),ERR1=8.43 (110%)
Model 2	OFV	Estimates (SE%)
CL, V1, Q, V2, 2etas Y=F+ERR(1)	579.823	CL=0.0151(0.291%); V1=1.08 (19.5%) Q=0.00723 (3.55%); V2= 0.0634 (23.1%) ETA1=0.439 (18.3%); ETA2=0.0802 (10.5%) ERR1= 8.28 (144%)
Model 3	OFV	Estimates (SE%)
Vm, Km, Vd 2etas (Km ,Vd); Y=F+ERR(1)	576.971	Vm=0.385 (2.51%); Km=8.74 (67.1%); Vd=1.15 (7.55%); ETA1=1.88 (123%); ETA2=0.0512 (2.52%); ERR1=7.99 (127%)
Model 4	OFV	Estimates (SE%)
CL, V, Vm, Km Y=F+ERR(1)	579.446	CL=0.0152 (0.702%);V=1.13 (10.7%) Vm=0.0105 (511%); Km=8530 (huge) ETA1=0.434 (60.9%); ETA2=0.0722 (3.90%) ERR1=8.29 (108%)

CL: L/hr/Kg; V: L/Kg; Vm: mg/kg/hr; Km: mg/L; Vd:L/kg

Table 6-2: Base model estimates for normothermia and hypothermia and combined data set

Model parameter Estimates (SE%)	Normothermia N=9	Hypothermia N=10	Combined base model N=19
Vm (mg/kg/hr)	0.362 (1.91%)	0.542 (41.4%)	0.385 (2.51%)
Km (mg/L)	5.04 (74.6%)	23.2 (3220%)	8.74 (67.1%)
Vd (L/kg)	1.17 (14.8%)	1.13 (7.49%)	1.15 (7.55%)
CL (L/kg/hr)	0.0718	0.0234	0.0441
$\omega$ 2 (Km)	5.70 (481%)	0.219(29.6%)	1.88(123%)
$\omega$ 2 (V1)	0.105 (5.57%)	0.011 (0.563%)	0.05(2.52%)
$\eta$ 1	7.20 (155%)	8.99 (222%)	7.99 (127%)

#### 6.4.2 Covariate model

The covariate model with temperature covariate on  $K_m$  has significantly reduced the OFV value 5.58 points from the best base model mentioned above ( $p < 0.05$ ). Hypothermia significantly increased the  $K_m$  of phenytoin. Hypothermia effect on  $K_m$  can be described as:  $K_m = 13.8 \times (TEM/37)^{-6.45}$ ,  $V_{max} = 0.458$  mg/kg/hr, and  $V_d = 1.23$  L/kg. Every one degree decrease from 37°C, there is expected around 19.3%  $K_m$  increase for phenytoin. The individual  $K_m$  generated have been plotted with temperature in Figure 6-5. Body temperature at 33°C, the  $K_m$  of phenytoin will increase almost 2 fold (Table 6-3). Increased  $K_m$  indicated the reduced affinity from phenytoin to the metabolic enzyme. Therefore, hypothermia reduced the affinity of the substrates to the enzyme and reduced the elimination.

The covariate model with temperature covariate effect on  $V_{max}$  has also significantly reduced the OFV value 7.99 points from the best base model mentioned above ( $p < 0.01$ ). Hypothermia significantly decreased the  $V_{max}$  of phenytoin. Hypothermia effect on  $V_{max}$  can be described as:  $V_m = 0.424 \times (TEM/37)^{2.99}$ ,  $K_m = 11.2$  mg/L, and  $V_1 = 1.23$  L/kg. Every one degree decrease from 37°C, there is expected around 7.9%  $V_{max}$  decrease for phenytoin. Body temperature at 33°C, the  $V_{max}$  of phenytoin is expected to decrease 30% (Table 6-4).

When tested individually, both  $K_m$  and  $V_{max}$  have been affected by hypothermia, however, when temperature has been added to both  $K_m$  and  $V_{max}$  at the same time, the model did not reduce OFV further, this indicated a  $V_{max}$ - $K_m$  interactions. Based on the higher percentage changes per degree, we chose the temperature effect on  $K_m$  to continue. In addition, the intrinsic clearance of phenytoin ( $V_{max}/K_m$ ) has then been generated, and temperature-clearance

relationship has been described in Figure 6-6. Hypothermia decreased the clearance of phenytoin in pediatric TBI patients.

Table 6-3: Km change with temperature

Baseline 37°C	Km expected to increase to	The change	Fold increase
36°C	119.3%	+19.3%	Around 40% increase Around 70% increase Almost 2 fold increase More than 2 fold increase
35°C	143.1%	+43.1%	
34°C	172.5%	+72.5%	
33°C	209.1%	+109.1%	
32°C	255.1%	+155.1%	

$K_m = 13.8 \times (T_{EM}/37)^{-6.45}$ , Temperature on  $K_m$ ,  $p < 0.05$ ; Hypothermia (lower body temperature) increase  $K_m$  of phenytoin.

Table 6-4: Vmax change with temperature

Baseline 37°C	Vmax expected to decrease to	The change
36°C	92.1%	-7.90%
35°C	84.6%	-15.4%
34°C	77.6%	-22.4%
33°C	70.7%	-29.3%
32°C	64.7%	-36.3%

$V_{max} = 0.424 \times (T_{EM}/37)^{2.99}$ ; Temperature effect on  $V_{max}$ ,  $p < 0.05$ ; Hypothermia (lower body temperature) decrease  $V_{max}$  of phenytoin

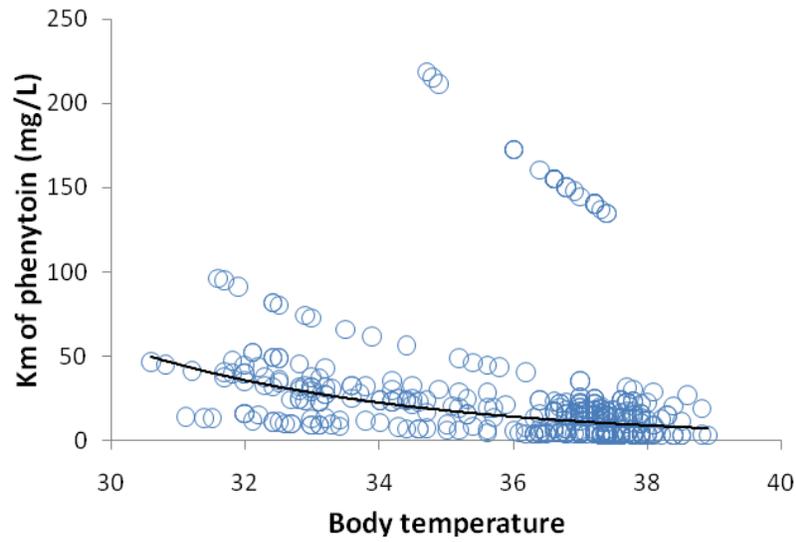


Figure 6-5: Body temperature-estimated Km curve. Hypothermia increases Km of phenytoin in pediatric traumatic brain injury patients

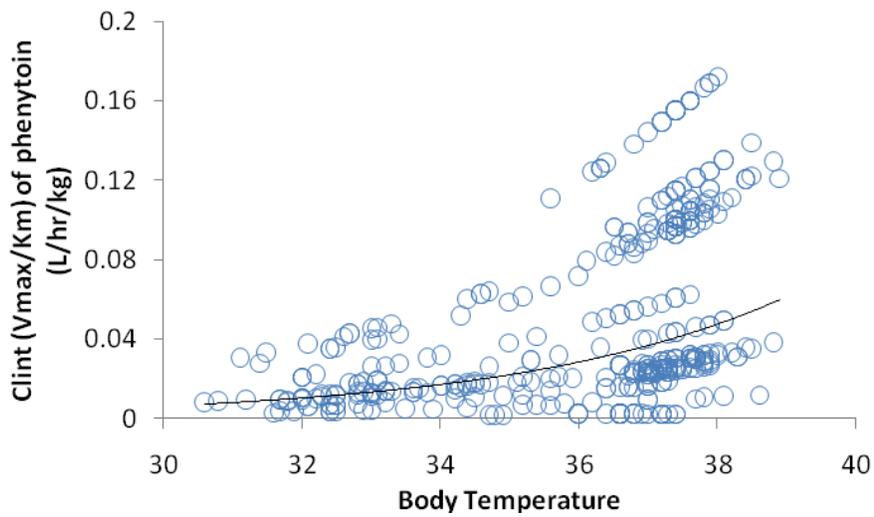


Figure 6-6: Body temperature-estimated clearance ( $V_{max}/K_m$ ) curves. Hypothermia decrease clearance in in pediatric traumatic brain injury patients

### 6.4.3 Final model evaluation

Estimated parameters with se% from the base model and the final model were listed in Table 6-5. All diagnostic plots revealed that the model was consistent with the observed data (Figure 6-7). Final model estimates for the structural and random variance parameter demonstrated larger inter-individual variability. The final model was assessed further by nonparametric bootstrapping method, the results of bootstrap estimates were consistent with NONMEM estimates which means the final model was relatively stable and robust (Table 6-5). Individual data with model fitting has also been shown in Figure 6-8. Model prediction is in great agreement with the individual observed data, therefore, the final model is also relatively accurate and reliable.

Table 6-5: Population PK parameters for phenytoin in pediatric TBI patients

Parameter Estimates (SE%)	Base model	Final model	Bootstrap Median (25-75% percentile)
Vm (mg/kg/hr)	0.385 (2.51%)	0.458 (5.06%)	0.478 (0.389-0.733)
Km (mg/L)	8.74 (67.1%)	13.8 (36.8%)	22.60 (8.03-66.80) (with $\theta_4$ )
$\theta_4$ (TEM/37)	--	(-6.45) (96.3%)	--
Vd (L/kg)	1.15 (7.55%)	1.23 (9.53%)	1.240 (1.12-1.39)
$\epsilon_2$ (Km)	1.88 (123%)	1.14 (77.6%)	-0.015 (-0.692-6.34)
$\epsilon_2$ (Vd)	0.0512 (2.52%)	0.0543 (3.08%)	0.00429 (-0.00758-0.6830)
$\eta_1$ (Err1)	7.99 (127%)	7.76 (119%)	7.89 (2.78-12.5)

Vm: maximum rate, mg/kg/hr; Km: Michaelis constant, mg/L; Vd: volume of distribution, L/kg TEM/37: temperature normalized by 37;  $\epsilon_2$ : inter-individual variability;  $\eta_1$ : residual error

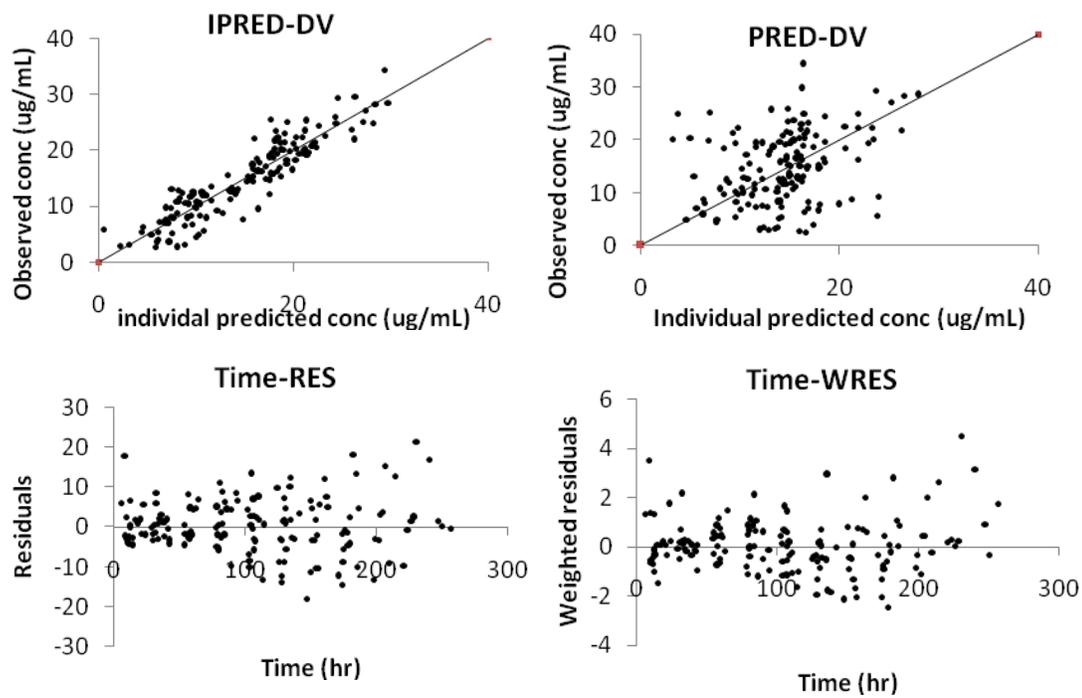


Figure 6-7: Goodness of fit plots for the final model of phenytoin in pediatric traumatic brain injury patients. The dashed lines in the upper panels are lines of unity

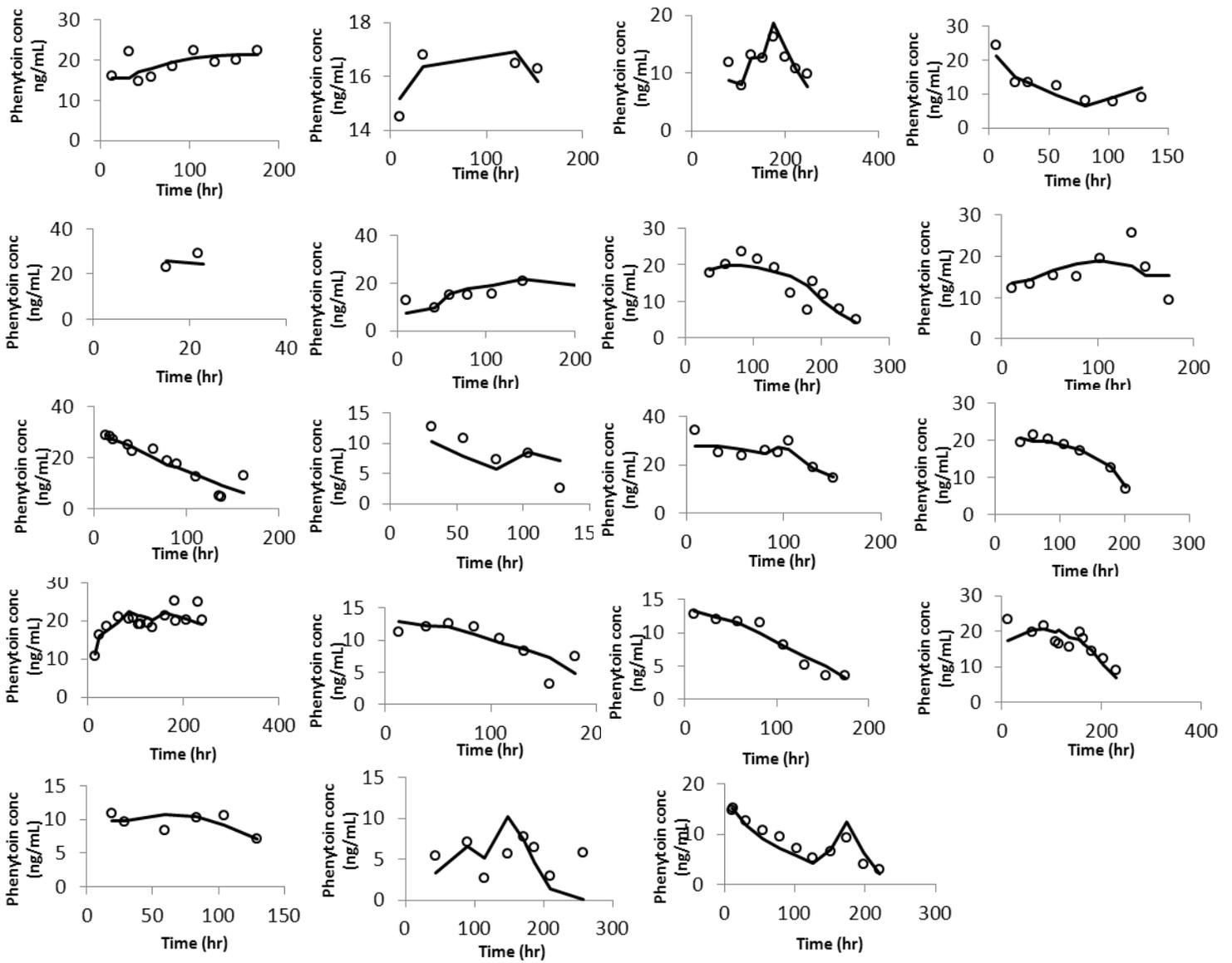


Figure 6-8: Individual time-concentration curves. The dotted points are observed values and the lines are model prediction value

#### 6.4.4 Temperature and protein binding

A correlation/collinearity analysis by S-plus 8.1 (TIBCO software Inc, CA) was tested for the possible correlation between temperature and unbound fraction (Fu). No correlation was found between temperature and Fu fraction, temperature did not change Fu (Figure 6-9). This result is consistent with previous studies which showed no protein binding change during hypothermia (Iida et al., 2001; Tortorici et al., 2006). Therefore, metabolism of drugs which are depends on hepatic enzyme activities would only be affected when hepatic enzyme activities are changed, regardless of high or low protein binding fraction.

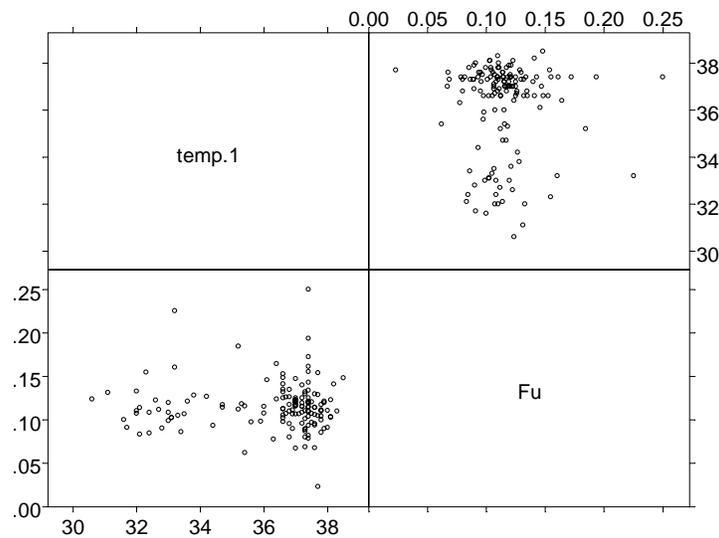


Figure 6-9: There is no correlation between temperature and unbound fraction

## 6.4.5 Simulation

### 6.4.5.1 Temperature curve

Temperature profile used in simulation has been divided into four different periods. (Figure 6-10):

A): Time 0-6 hours  $Y = -0.6667X + 37$  ( X is the time point, Y is the temperature)

(follows protocol induction from 37-33°C in 6 hours, based on linear temperature change).

B): Time 6-52 hour: maintain at 33°C

C): Time 52-100 hour  $Y = 0.0833X + 28.667$

(follows protocol rewarming from 33-37°C in 48 hours, based on linear temperature change)

or: Time 52-148 hour  $Y = 0.0417X + 30.833$

(follows protocol rewarming from 33-37°C in 96 hours, based on linear temperature change)

D): Time 100-200 hour, maintain at 33°C

or: Time 148-200 hour, maintain at 33°C

### 6.4.5.2 Simulated time-concentration curves in three groups

Based on the final model estimates, simulations for a single patient in hypothermia with 48 hrs rewarming, a single patient in hypothermia with 96 hrs rewarming and a single patient in normothermia have been done. Besides temperature covariate, phenytoin dose, study duration, has been provided in the following:

- 1) Simulation duration 0-200 hours.
- 2) Dose: 20mg/kg for loading dose, then 2.5mg/kg for every 12 hours

The simulation results have been shown in Figure 6-11. The patients in hypothermia showed significant elevated concentrations at later time points compared to those in the patient of

normothermia. More concentrations higher than 20ng/mL are observed. Phenytoin concentrations in 96 hrs rewarming group are slightly higher compared to 48 hrs rewarming group in later time points. Therefore, this significant comparison showed that hypothermia prolonged the elimination of phenytoin in pediatric patients. Slower rewarming may accumulated more drug in the body, however may not be significant.

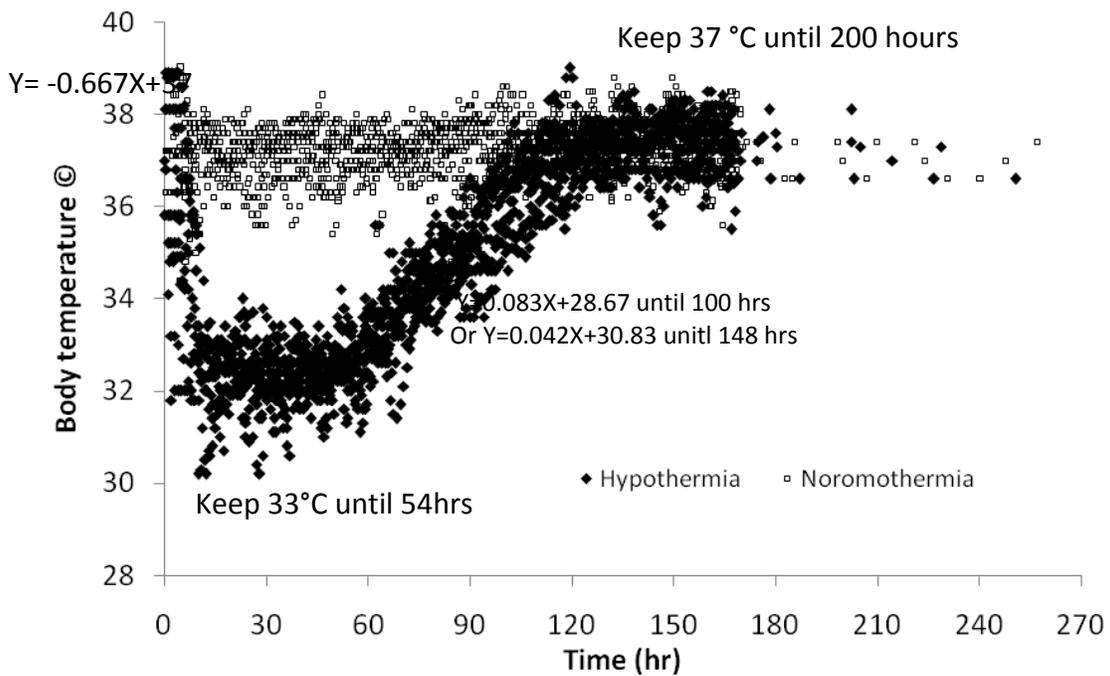


Figure 6-10: Temperature curve used for the temperature covariate in the simulation

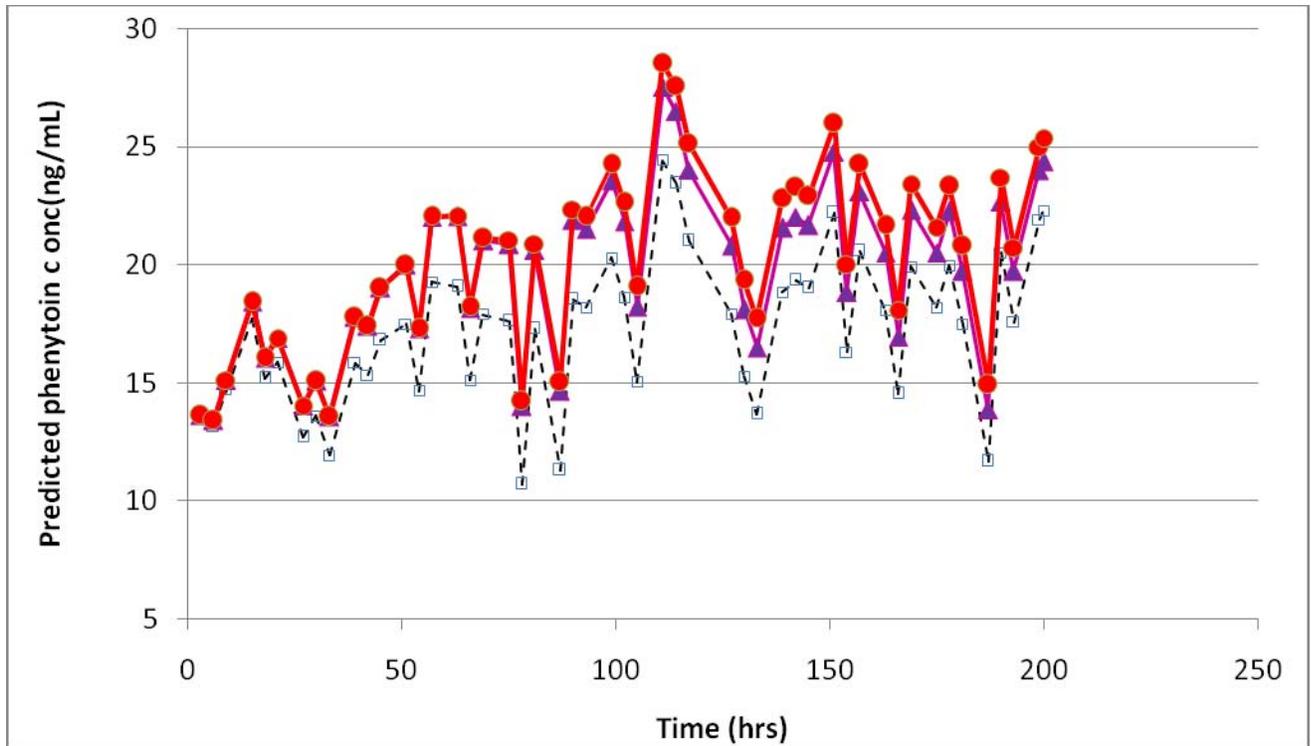


Figure 6-11: Final simulated time concentration profile from 0-200 hours. Three groups have been compared with the same and repeated dose regimen every 12hours (dotted line with blank square =normothermia; solid line with triangle=hypothermia with 48hrs rewarming; solid line with solid circle =hypothermia with 96hrs rewarming). Phenytoin concentrations in hypothermia with 48 hours rewarming and hypothermia with 96 hours rewarming group are higher than normothermia group. In addition, the concentrations in 96 hrs rewarming group are higher compared to 48 hrs rewarming group at later time points.

## 6.5 CONCLUSION AND DISCUSSION

The results of this study showed us hypothermia cooling for 48 hours significantly affected phenytoin PK in pediatric patients. Compared with previous literature, phenytoin PK estimates in this study is consistent with other studies (Blain et al., 1981; Berg et al., 1987; Levine et al., 1987). During hypothermia, previous study has reported the reduced clearance of phenytoin of 67% (Iida et al., 2001). Phenytoin is metabolized by CYP2C9 and CYP2C19. Our previous cardiac arrest animal data has shown hypothermia reduced the clearance of CYP2C9 substrate diclofenac (Chapter 3). In addition, this effect has interaction with volume of distribution. This data showed the reduced intrinsic clearance ( $V_{max}/K_m$ ) of phenytoin, and hypothermia did not affect unbound fraction of phenytoin, therefore, the activity of CYP2C9 is likely reduced during hypothermia in patients.

Hypothermia significantly increases  $K_m$  of phenytoin. Every one degree decrease from 37°C, there is expected around 19.3%  $K_m$  increase for phenytoin. The results indicated that hypothermia decreased the affinity from substrates to enzyme, and led to the decreased elimination and clearance. Predictive simulation also confirmed that hypothermia reduced phenytoin metabolism and elimination. In addition, slower rewarming at rate of 1 degree over 24 hours resulted in higher concentrations of phenytoin in later time points compared to the group with rewarming rate at 1 degree over 12 hours. The results and method can be used to predict individual patients time concentration profile, therefore will provide the response prediction and appropriate drug use guidance. The specific relationship derived from population modeling can be used as guidance in estimating and predicting of metabolism changes and appropriate dosage

for drug safety and efficacy. More study using the same study design and population in different temperatures, different doses, and different probe drugs are needed to fully validate these results.

The net effect of these increases in drug concentrations is likely to accumulate during rewarming as seen in the simulation. Depending on the half-life and dose frequency of the given drugs, the drug concentrations will remain elevated until a new steady state is established well into the rewarming period, whereas, full receptor response may return rapidly upon temperature normalization. Phenytoin has a long half-life of around 22 hours, and 3-5 half-lives of 3 to 6 days, therefore it will take a longer rewarming time for the accumulated drug to be eliminated from the body. The altered pharmacokinetics cannot be simply translated into the altered efficacy or toxicity since the drug response and pharmacodynamics is not available. Further studies on drug receptor and PK-PD relationships with temperature and diligent clinical monitoring are necessary.

## 7.0 SUMMARIES AND FUTURE DIRECTION

Therapeutic hypothermia is a proven neuroprotective strategy in multiple animal models and in patient studies for both adults out of hospital cardiac arrest and neonates with hypoxic ischemic encephalopathy. Clinical studies demonstrating neurological benefits have cooled adult patients to 34 to 32°C for 12 to 24 hours, whereas neonates were cooled over the same temperature range for up to 72 hours. Patients who survived to the point of inpatient care are most vulnerable to succumb to this disease during the 24 to 72 hours in which a patient is being cooled and during rewarming period. A wide array of pharmacologic agents are used in these patients, many of which have significant toxicities that may affect ventilator weaning, ability to assess seizure control, assessment of neurocognitive function, and hypotension. Furthermore, many of the enzymes and transporters that affect drug disposition are temperature sensitive and energy dependent. Despite this potential of drug-therapy interaction, few studies have been conducted to evaluate the clinical effects of therapeutic hypothermia on drug metabolism and mechanisms, and there was little report on dosing algorithms for patients during cooling and after rewarming. Based on this need, it is critical for currently ongoing experiments and randomized control trials aimed at evaluating the efficacy of therapeutic hypothermia also include sample collection for assessment of drug concentrations as part of the study design.

There are several conclusions can be made based on our clinical and preclinical results.

- Mild hypothermia and cardiac arrest alter CYP activity in an isoform specific manner
- Magnitude of the reduction is likely temperature, and extraction ratio specific

- Short duration hypothermia studies with hepatically eliminated drugs suggest ~11% reduction in clearance per °C change
- Microdosed PK likely to be very useful in any PK design in critically ill patients
- Therapeutic hypothermia inhibited phenytoin metabolism,  $K_m$  increase ~19% per °C
- Effect of cooling on receptor dynamic response is unknown on current data

In the current study, we did not study any flow dependent drugs. Based on previous literature, similar to low extraction drugs, the metabolism of high extraction ratio drugs also appear to be decreased during hypothermia, although the magnitude of flow alterations is highly temperature dependent and is likely greater in magnitude at temperatures below 32°C. A limited number of preclinical and in vitro studies suggest that active tubular secretion and drug transporter activity are also reduced during therapeutic hypothermia. In contrast, passive renal filtration and drug protein binding do not appear to be significantly altered during cooling.

The aforementioned effects of therapeutic hypothermia on drug metabolism have been supported by clinical studies demonstrating increased drug concentrations in patients during the cooling period. Although drug concentrations are elevated, it is important to recognize that increased concentrations may or may not be translated into changes in drug response. Response to certain drugs, such as vecuronium was not altered during cooling; therefore, elevated concentrations translate into a prolonged duration of effect. Conversely, in vitro studies have demonstrated significant reductions in morphine receptor response under hypothermic temperatures, thereby, suggesting a potential decrease in response despite increases in circulating morphine concentrations. Drugs with reduced drug responsivity during cooling will be particularly

problematic in critically ill patients because many of these drugs are dosed to clinical response. This results in an increased dosing despite overall decreased elimination resulting in further elevations in drug concentrations during cooling.

Previous literature and our current study results demonstrated the need for close monitoring of the drugs administration not only in hypothermic condition but also in normothermic condition in clinical practice. The reduction in hepatic blood flow, altered gastrointestinal absorption and diminished intrinsic hepatic metabolic activity after cardiac arrest can directly associated with altered drug concentration and metabolism. The pharmacokinetics of many drugs used to treat heart failure and critical care patients can be significantly altered by the patients underlying condition. Up to now, guidelines to dosage adjustments on cardiac arrest and heart failure patients is incomplete, and this is tends to be overlooked in clinical practice. Further studies focus on cardiovascular specific drugs and patients whose conditions fluctuate widely are necessary.

When two factors coexist and have potential interaction, for example, the hypothermia and the cardiac arrest factors for cardiac arrest patients receiving hypothermia, to estimate the changes of drug metabolism, response and make clinical adjustment is often very difficult. From our results, use drugs that are metabolized by CYP3A should always be cautious since both hypothermia and CA can affect the drug metabolism and distribution. Use drugs have narrow therapeutic windows should warrant a close clinically monitoring. Use drugs with a longer half life should be kept in mind that the accumulated drug levels may remain longer than expected. Carefully recording all the variables and further population modeling and simulations are needed to test and define the

significant variables and association. Larger sample size study is necessary to make the accurate estimates as well as the validation of the established model. Additionally, studies in smaller groups of well matched patients, evaluating probe drug concentrations will provide additional important information. Before a general rule can be concluded, start investigating the specific individual clinical relevant drugs as certain model drugs might be more relevant in clinical practice and safety.

In closing, it is clear that a great deal of additional research is needed before a full understanding of the magnitude of the effects of therapeutic hypothermia on drug concentrations and responses. This void in our knowledge can be filled with the incorporation of drug concentration studies within currently ongoing clinical trials in patients receiving therapeutic hypothermia. Ultimately, studies should incorporate pharmacometrics tools in large patient populations to determine specific dosing algorithms for drugs with notable toxicities to guide clinicians in optimal pharmaceutical care in this highly vulnerable patient population.

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Figure 4-9: Time-concentration simulation curves of midazolam (three dose iv bolus) at 36.5°C (square symbols), 34°C (circle symbols) and 32°C (triangle symbols) using NONMEM estimated parameters. The 32°C profile has the highest AUC and C<sub>max</sub> followed by 34°C and 36.5°C curves. The predicted midazolam clearance at 34°C decreased to 74.0% compared to the clearance of core temperature baseline 36.5°C, the predicted midazolam clearance of 32°C decreased to 57.2% compared to the clearance in 36.5°C

Figure 5-1: Solid phase extraction procedure for using HLB and MAX cartridges

Figure 5-2: Typical chromatography window from UPLC/MS/MS in one of the validation days. Above showed chromatographies are midazolam, metoprolol, midazolam-d<sub>4</sub>, metoprolol-d<sub>7</sub>, esomeprazole, 1-hydroxymidazolam, omeprazole-d<sub>3</sub> and 5-hydroxyomeprazole (from left to right, then up to down direction)

Figure 5-3: Microdosed simulation rationale

Figure 5-4: Timeline of the protocol (BD= blood draw). Numbers indicate hours

Figure 5-5: Time concentration profile of midazolam, esomeprazole, metoprolol and famotidine of Subject 1

Figure 6-1: Body temperature in hypothermia and normothermia group

Figure 6-2: Phenytoin time-concentration (total) of pediatric TBI patients

Figure 6-3: Phenytoin dose regiment for hypothermia and normothermia patients

Figure 6-4: One or two compartment model with nonlinear or first order linear structure for phenytoin base model

Figure 6-5: Body temperature-estimated  $K_m$  curve. Hypothermia increases  $K_m$  of phenytoin in pediatric traumatic brain injury patients

Figure 6-6: Body temperature-estimated clearance ( $V_{max}/K_m$ ) curves. Hypothermia decrease clearance in in pediatric traumatic brain injury patients

Figure 6-7: Goodness of fit plots for the final model of phenytoin in pediatric traumatic brain injury patients. The dashed lines in the upper panels are lines of unity

Figure 6-8: Individual time-concentration curves. The dotted points are observed values and the lines are model prediction value

Figure 6-9: There is no correlation between temperature and unbound fraction

Figure 6-10: Temperature curve used for the temperature covariate in the simulation

Figure 6-11: Final simulated time concentration profile from 0-200 hours. Three groups have been compared, phenytoin concentrations in hypothermia with 48 hours rewarming and hypothermia with 96 hours rewarming group are higher than normothermia group. In addition, the concentrations in 96 hrs rewarming group are higher compared to 48 hrs rewarming group at later time points.

## LIST OF ABBREVIATIONS

ABC: ATP binding cassette;	FOCE: First order conditional estimation method;
ACLS: Advanced cardiac life support;	Fu: Unbound fraction;
ATP: Adenosine triphosphate;	GFR: Glomerular filtration rate;
AUC: Area under curve;	GPCR: G-protein coupled receptor;
BMI: Body Mass Index;	HIE: Hypoxic ischemic encephalopathy;
CA: Cardiac arrest;	HLB: Hydrophilic-lipophilic balance cartridges;
cAMP: Cyclic adenosine monophosphate;	HR: Heart rate;
CI: Confident interval;	hrs: Hours;
CL: Systemic clearance;	IACUC: Institutional Animal Care and Use Committee;
CL'int: Intrinsic clearance;	IC50: The half maximal inhibitory concentration;
CLb: Bile clearance;	ICG: Indocyanine green;
CLh: Hepatic clearance;	ICU: Intensive care unit;
CNS: Central nerve system;	IL: Interleukin;
CO: Cardiac output;	IPRED: Individual predicted concentrations;
COMT: Catechol-O-methyltransferase;	LOD: Limit of detection;
Conc: Concentration;	LOQ: Limit of quantitation;
CSF: Cerebrospinal fluid;	IRB: Institutional review board;
CYP: Cytochrome P450;	IS: Internal standard;
ELISA: Enzyme linked immunosorbent assay;	IV: Intravenous;
ER: Extraction ratio;	Ke: Elimination rate;
FD-4: Fluorescein isothiocyanate (FITC)-dextran;	
FDA: Food and drug administration;	

K<sub>m</sub>: Michaelis-Menten constant;  
LV dp/dt<sub>max</sub>: Left ventricle contractility;  
NMDA: N-methyl-d-aspartate;  
M3G: Morphine-3-glucuronide;  
M6G: Morphine-6-glucuronide;  
MAC: Minimum alveolar concentration;  
MAP: Mean arterial blood pressure;  
MAO: Monoamine oxydase;  
MAX: Mixed mode anion exchange cartridges;  
mins: Minutes;  
MRP1: Multidrug resistance protein 1;  
MRP2: Multidrug resistance protein 2;  
MRT: Mean resident time;  
MW: Molecular weight;  
NONMEM: Nonlinear mixed effect (NLME) modeling;  
OATP: Organic anion transporter;  
OFV: Objective function value;  
PGP: P-glycoprotein;  
PRED: Population predicted concentrations;  
PK/PD: Pharmacokinetics/Pharmacodynamics;  
PSP: Phenolsulfonphthalein;  
POAH: preoptic-anterior/hypothalamus;  
Q: Blood flow;  
QC: Quality control sample;  
RT: Retention time;  
SE: Standard error;  
SPE: Solid phase extraction;  
SULT: Sulfotransferase;  
SV: Stroke volume;  
T<sub>1/2</sub>: Half-life;  
T<sub>3</sub>/T<sub>4</sub>: Local or circulation thyroid hormone;  
TAAR1 receptor: Trace amine associated receptor 1;  
TBI: Traumatic brain injury;  
TH: Therapeutic hypothermia;

UGT: UDP-galactose transporter;

V1: Volume of distribution in central  
compartment;

Vd: Volume of distribution;

Vss: Volume of distribution at steady state;

Vmax: Nonlinear elimination maximum  
velocity;

WRES: Weighted residual;

