The Effect of Therapeutic Hypothermia on Drug Metabolism and Transport: Clinical and Pre-Clinical Investigations

by

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ABSTRACT

Therapeutic hypothermia, active cooling of a patient to a core body temperature of 32-34°C, is a neuroprotective therapy that is employed in critically ill patients to prevent further neuronal damage following an acute injury. While undergoing therapeutic hypothermia, critically ill patients are also administered a multitude of medications, which puts them at high risk for adverse drug events. The majority of these drugs undergo hepatic elimination via cytochrome P450 (CYP450) metabolic pathways and/or many also undergo active transport via the ATP-Binding Cassette (ABC) drug transporter pathways. In order to safely and effectively treat this critically ill population, it is imperative to understand how therapeutic hypothermia effects drug metabolism and drug transport. Therefore, the overarching aim of this dissertation was to expand what is currently known about the effects of therapeutic hypothermia on CYP450-mediated drug metabolism in a pediatric clinical study evaluating phenytoin elimination and to pre-clinically investigate the effects of hypothermia on drug transport via the ATP-binding cassette drug transporter pathways. Specifically, therapeutic hypothermia led to an overall decrease in the metabolism of phenytoin by reducing the maximum velocity of the enzymatic reaction. Furthermore, therapeutic hypothermia led to a decrease in the active drug transport of three ATP-Binding Cassette transporters (ABC), which play a significant role in the transport of drugs in various tissues throughout the body. Specifically, hypothermia to 33°C led to a decrease of

ABCB1, ABCG2, and ABCC1 by 28, 32, and 26% in permeability flux assays in MDCKII overexpressed-cells.

Collectively, this work demonstrates that therapeutic hypothermia decreases drug metabolism in pediatrics following cardiac arrest. Further, it provides evidence of a decrease in ABC drug transport activity *in vitro*. Future research should investigate the effect of therapeutic hypothermia on drug transport activity in clinical studies. Additionally, studies should investigate the effects of therapeutic hypothermia versus injury/disease on drug pharmacokinetics in order to optimize the therapeutic benefits of medications by customizing dosing based on disease and therapeutic intervention in these critically ill patients.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

My experience in pursing the degree of Doctor of Philosophy in the Pharmaceutical Sciences at the University of Pittsburgh has been rewarding and outstanding. This is in large part due to a number of excellent mentors, colleagues, and peers I have had the pleasure of working with and learning from during the program. While it's not possible to thank all of the exceptional individuals who have played a role in my development to date, there are a number that I would like to acknowledge here.

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ABBREVIATIONS

ABC:	ATP-binding cassette
ADE:	adverse drug event
ADR:	adverse drug risk
AED:	anti-epileptic drugs
ALBL:	albumin
ALBH:	hepatic albumin
ALTH:	alanine aminotransferase
ASTH:	aspartate aminotransferase
AUC:	area under the curve
BCRP:	breast cancer resistance protein
BILIH:	bilirubin
BLQ:	below limit of quantification
BUNH:	blood urea nitrogen
CA:	cardiac arrest
CDER:	Center for Drug Evaluation and Research
Cl_s:	systemic clearance

Cl_{TOT}: total clearance

C_{max}: maximum concentration

C_{min}: minimum concentration

Concs: concentrations

CRH: corticotropin-releasing hormone

CV: coefficient of variation

CWRES: conditional weighted residuals

CYP450: cytochrome-P450

DV: dependent varaiable

ECMO: extra-corporeal membrane oxygenation

ER_a: efflux ratio; ratio of the initial rate of B→A flux divided by the initial rate of A→B flux

FDA: Food and Drug Administration

FITC: fluorescein isothiocyanate

FD-4: fluorescein isothiocyanate-dextran

GI: gastrointestinal

GoF: goodness-of-fit

HACA: hypothermia after cardiac arrest

HIE: hypoxic-ischemic encephalopathy

HPLC: high-performance liquid chromatography

HSP: heat-shock protein

ICG: indocyanine green

ICU: intensive care unit

IS: internal standard

ICP: intracranial pressure

IPRED: individual predicted

IRES: individual residuals

IWRES: individual weighted residual

K_m: Michaelis-Menten rate constant

k_e: elimination rate constant

LEV: levetiracetam

LEV-d_6: levetiracetam-d_6

LLOD: lower limit of detection

LLOQ: lower limit of quantification

MDCK: Madin-Darby canine kidney

MDCKII: Madin-Darby canine kidney-II

MDR1: multidrug resistance protein-1

MRP1: multidrug resistance related protein-1

MF: matrix factor

MS/MS: tandem mass spectrometry

NONMEM: nonlinear mixed-effects modeling

OCT: organic cation transporter

OFV: objective function value

P_{app}: observed permeability

PC: prediction corrected

pcVPC: prediction corrected visual predictive check

P-gp: p-glycoprotein

PLC: phospholipase C

PHY: phenytoin

PHY-d_{10}: phenytoin-d_{10}

PopPK: population pharmacokinetic

PsN: Perl-speaks NONMEM

PSP: phenolsulfonphthalein

QC: quality control

Q: inter-compartmental clearance

RCT: randomized control trial

RE: relative error

ROSC: return of spontaneous circulation

RSE: relative standard error of the estimate

SLC: solute carrier

$t_{1/2}$: elimination half-life

TBI: traumatic brain injury

TDM: therapeutic drug monitoring

TEER: transepithelial electrical resistance

TH: therapeutic hypothermia

TJ: tight junction

TTM: targeted temperature management

UPLC: ultra-performance liquid chromatography

V_1: volume of the central compartment

V_2: volume of the peripheral compartment

V_D: volume of distribution

V_{max}: maximum velocity of metabolism

VPC: visual predictive check

1.0 INTRODUCTION AND BACKGROUND

[Anderson KB, Kochanek PM, Poloyac SM, Empey PE. *Targeted Temperature Management*. Accepted Aug 2016.]

[Anderson KB, Poloyac SM. Drug Metabolism and Therapeutic Hypothermia. *Critical Connections.* June 2015.]

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1.1 THERAPEUTIC HYPOTHERMIA

This section provides a brief discussion on the background of therapeutic hypothermia (TH), its implementation and use in critical care, the optimal depth and duration for therapeutic cooling, the current recommended guidelines for TH, and the physiologic effects of TH.

1.1.1 Background on Therapeutic Hypothermia

Following an ischemic injury, critically ill patients are at high risk for clinical neurological injury, which contributes to overall morbidity and mortality in these patients (Narayan, Michel *et al.* 2002, Rea, Pearce *et al.* 2004, De Keyser, Uyttenboogaart *et al.* 2005). Approximately 200,000-500,000 patients have an out-of-hospital cardiac arrest each year (Rea, Pearce *et al.* 2004). In addition, approximately 16,000 pediatric patients suffer a CA each year in the United States (Binks and Nolan 2010, Tress, Kochanek *et al.* 2010). Of those that survive these severe initial injuries, neurological damage is a common sequelae leading to significant disability and lost cognitive function (Levy, Caronna *et al.* 1985, Jorgensen and Holm 1998). However, limited drug therapy options are available to provide neuro-protection and prevent secondary injury after resuscitation (Laver, Farrow *et al.* 2004).

Therapeutic hypothermia is a neuroprotective therapy that is employed to prevent neuronal damage following an acute injury (Karibe, Chen *et al.* 1994, Lei, Tan *et al.* 1994, Aibiki, Maekawa *et al.* 1999, Bernard, Gray *et al.* 2002). While the use of therapeutic hypothermia to prevent neuronal injury is not a new therapy, the implementation of therapeutic hypothermia has increased and evolved over the past two decades as new research has emerged.

From 2000 to 2010 the use of TH in the intensive care unit (ICU) increased largely as a result of two pivotal randomized control trials (RCTs), which demonstrated a decrease in mortality and an improvement in neurological outcome in patients experiencing out-of-hospital cardiac arrest (CA) (Bernard, Gray *et al.* 2002, Group 2002, Shankaran and Laptook 2007). The Hypothermia After Cardiac Arrest (HACA) study investigated the effect of therapeutic hypothermia (32 – 34°C for 24 hours) on neurological outcomes and mortality in resuscitated patients following a CA due to ventricular fibrillation. The HACA study showed improved neurological outcomes in out-of-hospital CA patients who were cooled as compared to those who were not cooled (Risk Ratio: 1.4, $p<0.009$). Further, hypothermic CA patients had a decrease in mortality as compared to the normothermic group (Risk Ratio = 0.74; confidence interval = 0.58-0.95). In another RCT, Bernard *et al.* also demonstrated an improvement in cardiovascular effects in out-of-hospital CA patients undergoing hypothermic treatment (33°C for 12 hours) as compared to the normothermic control group. Of the patients undergoing therapeutic hypothermia 49% were discharged to home or a rehabilitation facility, whereas only 26% of patients undergoing normothermia treatment were discharged ($p = 0.046$).

In addition to out-of-hospital adult CA patients, therapeutic hypothermia has also been shown to be efficacious in perinatal asphyxia (Shankaran, Laptook *et al.* 2005). In a RCT in infants with moderate or severe encephalopathy, Shankaran *et al.* demonstrated an improvement in the primary outcome measurement (death or moderate or severe disability) in infants who were randomly assigned to hypothermia treatment to 33.5°C for 72 hours, as compared to the standard-of-care normothermia control group. In the hypothermic group, death or moderate or severe disability occurred in 44% of the infants, in contrast to 62% in the normothermic group (risk ratio = 0.72; 95 percent confidence interval, 0.54 to 0.95; $p=0.01$).

To date, the two main patient populations that therapeutic hypothermia is recommended and used in are 1) out-of-hospital adult cardiac arrest patients and 2) neonates with hypoxic-ischemic encephalopathy (HIE). TH has also been studied in a number of other patient populations including traumatic brain injury (TBI) (Clifton, Valadka *et al.* 2011, Georgiou and Manara 2013, Beca, McSharry *et al.* 2015, Zhang, Wang *et al.* 2015, Cooper, Nichol *et al.* 2016), stroke (Kollmar, Schellinger *et al.* 2009, Bi, Ma *et al.* 2011, Wan, Nie *et al.* 2014), and spinal cord injury (Levi, Green *et al.* 2009, Grulova, Slovinska *et al.* 2013). Currently, TH is not used for neuroprotection in TBI but it is employed to control intracranial pressure (ICP). Moreover, conflicting evidence has led to varying recommendations in these patient populations and in some cases its benefit is outweighed by its adverse drug risk (ADR). As it pertains to this dissertation, understanding how to successfully implement therapeutic hypothermia by understanding its underlying effect on drug disposition is critical.

The use of therapeutic hypothermia as a neuroprotective treatment strategy in critically ill patients has shifted focus over the past decade from the initial pivotal RCTs in the early 2000s. Based on these initial RCTs, TH was recommended in the American Heart Association Guidelines for adult out-of-hospital CA patients and in neonates with hypoxic ischemic encephalopathy (Nolan, Morley *et al.* 2003). Subsequently, in the largest hypothermia RCT to date, no significant difference was seen in the neurological outcomes of adult CA patients who received therapeutic hypothermia (33°C) versus targeted temperature management (TTM) (36°C) (Nielsen, Wetterslev *et al.* 2013). These results have raised questions regarding the benefits of cooling to 33°C. Recently, Moler *et al.* (Moler, Silverstein *et al.* 2015) studied the effect of therapeutic hypothermia (33°C for 48 h) as compared to normothermia (36.8 °C) in children who suffered an out-of-hospital CA. A trend toward improved outcome (12 month

survival with favorable neurological outcome as assessed by the Vineland Adaptive Behavioral Scale) was seen with hypothermia versus normothermia (p=0.14) along with mortality (p=0.13). Given that the power of the study to detect the rather demanding target of 20% improvement in outcome was only 42%, some have suggested that this study supports the use of hypothermia in this population—until proven otherwise (Vincent and Taccone 2015). Thus, TTM at various levels of hypothermia, continues to be recommended for use in neonates with HIE and to treat out-of-hospital CA patients (Callaway, Donnino *et al.* 2015). As further evidence becomes available, the degree of which we cool patients may increase from the previous target clinical temperature of 32-34°C. Indeed, recent evidence indicates that even a 1°C reduction in temperature induces neuroprotective signaling cascades in neurons in *in vitro* systems (Jackson, Manole *et al.* 2015). However, studies are needed to delineate the specific patients who maximally benefit from hypothermia treatment based on additional considerations such as severity of injury, organ dysfunction following cardiac arrest, inflammation, and co-administered medications.

1.1.2 Implementation of Therapeutic Hypothermia

In a broad sense, therapeutic hypothermia is defined as a core body temperature less than 35.0˚C. Moreover, there are different degrees of hypothermia which incur a range of neuroprotection and adverse physiologic effects. Hypothermia can be divided based on the degree of cooling and include mild hypothermia, moderate hypothermia, and severe hypothermia. It is generally accepted that mild hypothermia occurs when a subject is cooled to a temperature of 32-34˚C whereas moderate hypothermia is at a temperature range of 30 – 32˚C. Severe, or "deep" hypothermia, is defined as cooling to a temperature below 30˚C.

Furthermore, therapeutic hypothermia undergoes different lengths of cooling depending on the patient population. Adult cardiac arrest patients typically undergo therapeutic hypothermia for 24-48 hours, whereas neonates with HIE are cooled for 72 hours. The AHA guidelines recommend that a minimum of 24 hours of cooling be achieved (Callaway, Donnino *et al.* 2015). The duration of cooling is largely based on the design of randomized control trials which demonstrated outcome benefits. Although these temperatures tend to be generally accepted, it is important to note that these categories can be arbitrary across studies and require verification of temperature and duration in the currently published literature. This dissertation will focus predominately on the effects seen within mild hypothermia (32 - 34˚C), since this is the clinically relevant temperature range that has been shown to afford neuroprotection without adverse physiologic consequences to critically ill patients in the ICU.

In addition to the duration of cooling, the time of initiation is also an important consideration. Therapeutic hypothermia should be started as soon as possible following return of spontaneous circulation (ROSC). However, studies have demonstrated that it appears to be successful even if it is initiated after 4 to 6 hours (Nolan, Morley *et al.* 2003). For example, in the HACA trial, the time between ROSC and reaching the target clinical temperature ranged from 4 to 16 hours (Group 2002).

There are a number of different cooling methods, such as external and internal techniques, which can be used to achieve target temperature (Nolan, Morley *et al.* 2003). External cooling methods consist of applying ice packs, wet towels, a cooling helmet, or a cooling blanket. These external methods are convenient and simple to use but often require a long time to achieve desired temperature. Internal methods of cooling such as intravenous infusion of cold saline have also been used to reach target body temperature. Moreover, careful

monitoring and control of temperature is essential when employing therapeutic hypothermia. Shivering must be controlled during cooling to prevent the body from rewarming. To control shivering, a neuromuscular blocking agent and sedative are employed. This is one example where drug-therapy interactions may occur during cooling.

1.1.3 Guidelines for the Use of Therapeutic Hypothermia

The recommendations for use of therapeutic hypothermia have shifted to reflect new clinical evidence in the field. In 2003 the International Liaison Committee on Resuscitation recommended 12-24 hours of cooling to 32-34°C in adult patients with spontaneous circulation with out-of-hospital cardiac arrest (Nolan, Morley *et al.* 2003). At this time, they suggested that such cooling may also be beneficial for in-hospital patients or for other rhythms. No recommendations were made for children as there was insufficient clinical data to support cooling in pediatric patients (Nolan, Morley *et al.* 2003).

In 2010, the guidelines recommended inducing therapeutic hypothermia for V-fib cardiac arrest patients (Morrison, Deakin *et al.* 2010). A cooling duration of 12-24 hours to a degree of 32-34°C was recommended. Most recently in 2015 the guidelines for therapeutic hypothermia changed drastically based on several large RCTs which investigated various target temperatures and durations (Callaway, Donnino *et al.* 2015). It was at this time that a newer term "targeted temperature management" was introduced to reflect the varying degrees of temperatures being used. Target temperature management became more commonly used to refer to both therapeutic hypothermia and to actively controlling temperature to any degree. These guidelines had three important recommendations. The first update was that TTM should be induced in comatose adult patients. Further, the degree of cooling was expanded to include recommendations for any

temperature between 32 – 36°C during TTM. Finally it was recommended that TTM be maintained for at least 24 hours after target temperature was achieved.

1.1.4 Physiologic Effects of Hypothermia

Before discussing the specific effects of therapeutic hypothermia on drug disposition and response, it is important to first recognize the general physiologic changes that occur in therapeutic hypothermia patients during the induction, maintenance, and rewarming phases. **Figure 1** depicts the known effects of therapeutic hypothermia on drug absorption, distribution, metabolism, excretion and response. Additionally, the quality of the current data (preclinical versus clinical evidence) is described.

1.1.4.1 Cardiovascular Effects of Hypothermia

The effect of therapeutic hypothermia on the cardiovascular system has been divided into two main areas 1) hemodynamic effects and 2) electrocardiographic effects. Both of which are described below.

Hemodynamic Effects

Hypothermia has been linked to changes in myocardial function. Mild hypothermia induces a decrease in heart rate, but produces an overall increase in the contractility of the heart in sedated patients (Goldberg 1958, Suga, Goto *et al.* 1988, Mikane, Araki *et al.* 1999, Lewis, Al-Khalidi *et al.* 2002, Fischer, Cox *et al.* 2005, Polderman and Herold 2009). Systolic function will improve, but diastolic function may decrease (Polderman and Herold 2009). Some patients may experience an increase in blood pressure while others may see no change in blood pressure. Overall, cardiac output will decrease along with the heart rate. However, the subsequent

hypothermia-induced decrease in metabolic demand tends to equal or exceed the decrease in cardiac output, thus keeping the balance between supply and demand constant (Polderman 2009).

In some cases, the heart rate may be artificially increased by drugs or external pacing. However, the effect of hypothermia on myocardial contractility has convoluted results under artificial stimulation (Mattheussen, Mubagwa *et al.* 1996, Mikane, Araki *et al.* 1999, Lewis, Al-Khalidi *et al.* 2002). Two pre-clinical studies showed that under normothermic conditions an increase in heart rate led to an increase in cardiac output and myocardial contractility (Mattheussen, Mubagwa *et al.* 1996, Mikane, Araki *et al.* 1999). In contrast, when heart rate was increased under mild hypothermic conditions there was a decrease in myocardial contractility. The same results were reported in a clinical study in patients undergoing cardiac surgery (Lewis, Al-Khalidi *et al.* 2002). When heart rate was not increased artificially, mild hypothermia improved myocardial contractility. Thus, in most patients heart rate should be allowed to decrease with temperature without any serious adverse complications.

Electrocardiographic Effects

Mild hypothermia has also been associated with abnormal heart rhythms. During cooling, hypothermia causes an increase in plasma norepinephrine levels and activation of the sympathetic nervous system. This leads to constriction of peripheral vessels and a shift of the blood from small, peripheral veins to centrally located veins in the core compartment of the body. Ultimately, this results in an increase in venous return which leads to mild sinus tachycardia. As temperature continues to drop even further below 35˚C, the heart rate begins to slow to a below normal rate eventually leading to what is known as sinus bradycardia. The heart rate will continue to decrease progressively as temperature drops to 33˚C and below (Danzl and Pozos 1994). The mechanism behind this is a decrease in the rate of spontaneous depolarization

of cardiac cells in combination with prolonged duration of action potentials. These electrocardiogram changes usually do not require treatment and in most cases a patient's heart rate should be allowed to decrease with cooling. Furthermore, some studies have linked hypothermia to an increased risk for arrhythmias. However, hypothermia-induced arrhythmias generally only apply to moderate to deep hypothermia, particularly when temperatures reach less than 30°C. During deep hypothermia, a patient is at higher risk to develop atrial fibrillation or ventricular fibrillation if temperatures reach as low as 28°C. Since temperatures are maintained at greater than 30°C in the ICU, few cases of hypothermia-induced arrhythmias have been observed in clinical trials evaluating the safety of mild therapeutic hypothermia (Polderman 2009).

1.1.4.2 Renal Effects of Hypothermia

Therapeutic hypothermia also has physiologic effects on renal function. During cooling, an increase in urinary output, known as cold diuresis, may occur. Cold diuresis results from a combination of an increase in venous return, a decrease in antidiuretic hormone, tubular dysfunction, and decreased levels of antidiuretic hormone and renal antidiuretic hormone receptor levels (Morgan, Anderson *et al.* 1983, Allen and Gellai 1993, Polderman, Peerdeman *et al.* 2001, Polderman, Tjong Tjin Joe *et al.* 2002, Sun, Zhang *et al.* 2003, Sun 2006).

Renal elimination can be divided into passive filtration, active tubular secretion and active tubular reabsorption. Passive glomerular filtration does not seem to be affected by therapeutic hypothermia. One clinical study investigated the effects of mild hypothermia on renal filtration by measuring serum creatinine levels and creatinine clearance in subjects with and without hypothermic treatment. The study found no change in creatinine clearance between the two

groups and concluded that cooling does not impair renal filtration (Zeiner, Sunder-Plassmann *et al.* 2004).

Although passive processes of renal filtration do not seem to be significantly altered, some published evidence does suggest that the active processes of tubular secretion and reabsorption may be altered by mild hypothermia (Nishida, Okazaki *et al.* 2007, Liu, Borooah *et al.* 2009). To date, the effect of therapeutic hypothermia on the active process of tubular secretion has only been studied preclinically in rats. This study used fluorescein isothiocyanate (FITC)-dextran to measure glomerular filtration and phenolsulfonphthalein (PSP) to measure renal tubular secretion in mildly hypothermic versus normothermic rats (Nishida, Okazaki *et al.* 2007). The results showed no change in FITC-dextran clearance, but a significant change in the renal clearance of PSP. These results provide further evidence that the passive process of renal filtration is unaffected by mild hypothermia, whereas, active renal tubular secretion is decreased during cooling. There are, however, a limited number of studies published to date and whether or not these initial evaluations remain true clinically will depend on more extensive assessments of the effects of mild hypothermia on renal drug elimination processes.

1.1.4.3 Electrolyte Effects of Hypothermia

Therapeutic hypothermia also alters electrolyte levels such as magnesium, potassium, and phosphate. During cooling, electrolytes shift from the bloodstream to the intracellular compartment. The low level of electrolytes remaining in the bloodstream increases a patients risk for hypokalemia. During rewarming, the opposite effect is seen and potassium, as well as other electrolytes, is released back into the bloodstream from the intracellular compartment. If the patient is rewarmed too quickly, potassium levels will increase abruptly in the bloodstream and the patient may become hyperkalemic. To avoid hyperkalemia, a slow and consistent rewarming

period is necessary to allow the kidneys to excrete the excess potassium. Furthermore, frequent lab electrolyte assessments are needed to account for shifts in systemic electrolyte concentrations.

1.1.4.4 Body Metabolism & Drug Clearance Effects of Hypothermia

Hypothermia has been shown to decrease the metabolic rate by approximately 8% per 1˚C drop in body temperature. A similar decrease in oxygen consumption and carbon dioxide production is observed. This decrease in metabolic rate arises from a global decrease in the rate of drug metabolism by the liver because the majority of the metabolic reactions in the liver are enzyme-mediated. The rate of these enzyme-mediated reactions is highly temperature sensitive; thus the rate of these reactions is significantly slowed during hypothermia. Hypothermia-induced reductions in clearance have been shown for a number of commonly used ICU sedatives such as propofol; opiates such as fentanyl and morphine; midazolam; neuromuscular blocking agents such as vecuronium and rocuronium; and other drugs such as phenytoin (Polderman 2009). The specific alterations in drug metabolism and clearance will be further addressed in more detail in the subsequent sections.

1.1.4.5 Gastrointestinal Effects of Hypothermia

Gastrointestinal (GI) motility decreases with mild hypothermia. In some cases, decreased motility leads to mild ileus which typically occurs at temperatures less than 32°C (Danzl and Pozos 1994). Other physiological factors play a large role in the extent to which drugs and nutrients are absorbed across the gut wall. As with drug excretion in the kidney, drug absorption across the intestinal membranes depends primarily on passive diffusion with significant contribution by active transport mechanisms for some drugs. Also similar to the kidney, cooling

was shown to affect active drug transport via the ABCB1 transporter, more commonly known as P-glycoprotein, *in vitro*. However, no effect of cooling has been reported on passive diffusion, thereby, suggesting that passive processes are unaltered and active drug transport may be impaired during cooling. Further physiological factors that affect absorption include the pH of various biological compartments and the blood flow at the site of absorption. The physiochemical properties of the drug, such as its pKa and lipid solubility, in combination with the compartmental pH, will influence the extent of which the drug will distribute into a given compartment. It is expected that some drugs will have increased absorption while others may have decreased absorption during cooling depending on pH, lipophilicity, and primary site of GI absorption; however, no studies to date have thoroughly evaluated if these anticipated changes occur *in vivo* under mild hypothermic conditions.

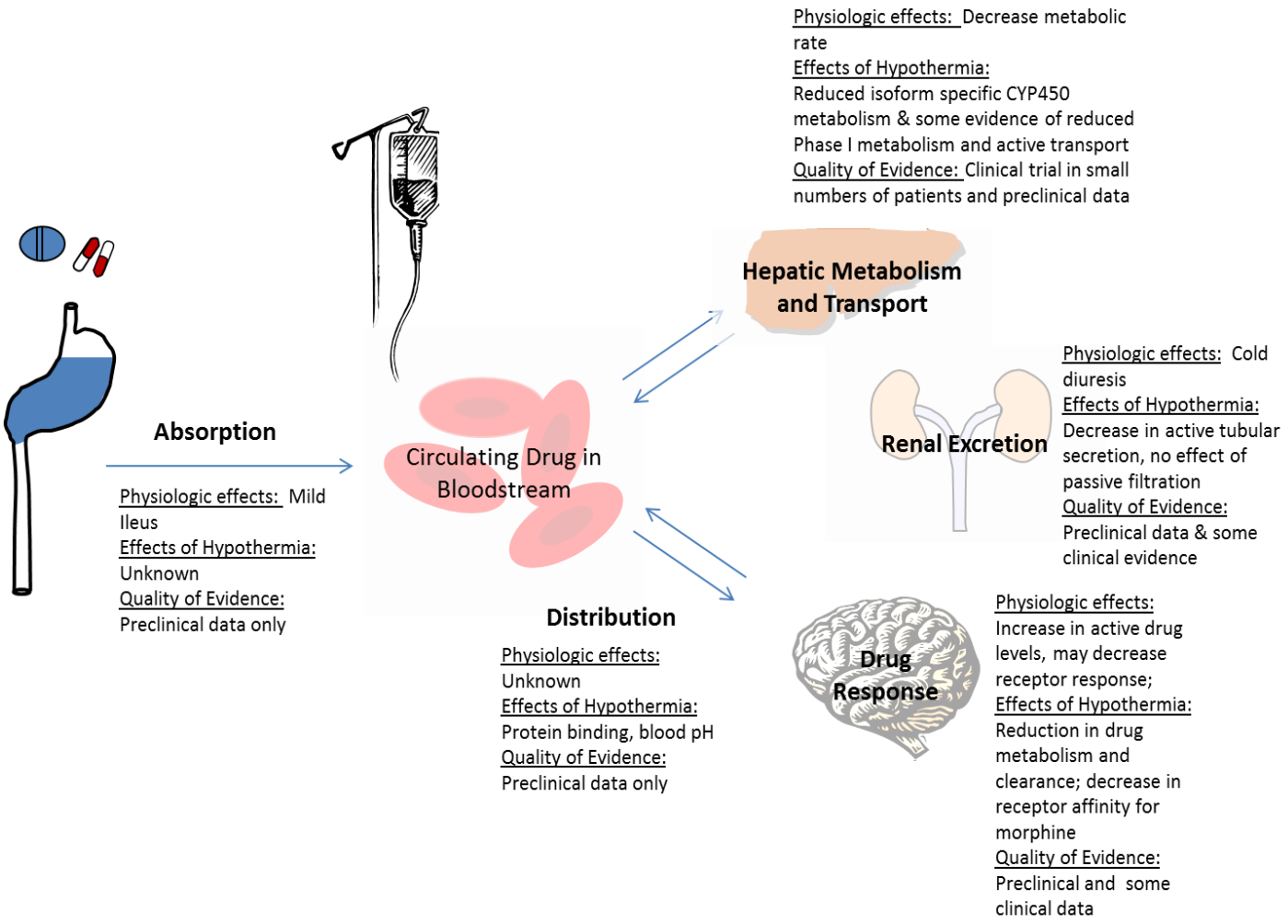


Figure 1: The effects of therapeutic hypothermia on drug absorption, distribution, metabolism/transport, renal excretion and drug response.

1.2 ADVERSE DRUG EVENTS IN CRITICAL CARE

The question of altered drug disposition and response in patients receiving therapeutic hypothermia is particularly important to the optimization of the wide array of drugs used in critically ill patients. This section briefly highlights some of the high risks associated with adverse drug events in critically ill patients, the commonly administered drugs in critical care, and the metabolic and transporter pathways associated with these medications.

1.2.1 Critically Ill Patients are at High Risk for Adverse Drug Events

Adverse drug events (ADE) are a significant problem among critically ill patients in the ICU (Kane-Gill, Rea *et al.* 2006, Barletta, Cooper *et al.* 2010, Devlin, Mallow-Corbett *et al.* 2010, Kane-Gill, Jacobi *et al.* 2010, Kane-Gill, Kowiatek *et al.* 2010, Papadopoulos and Smithburger 2010, Kane-Gill, Kirisci *et al.* 2012). In the ICU, serious ADEs occur in 26% of patients as compared to only 11% in non-ICU patients (Cullen, Sweitzer *et al.* 1997). The reason for this high rate of ADEs is due, in part, to the plethora of medications administered to patients for analgesia/sedation, paralysis, control of seizure activity, blood pressure, treatment of arrhythmias, control of blood clotting, antibiotics, and delirium prevention, many of which are administered simultaneously. **Table 1** provides a list of commonly administered drugs in the ICU organized by route of elimination. The high number of medications in combination with acute changes in organ function puts this vulnerable population at high risk for ADEs (Cullen, Sweitzer *et al.* 1997). Ultimately, these events may lead to extended sickness, mortality, and increased hospital costs (Vargas, Terleira *et al.* 2003).

Table 1: Commonly used medications in the ICU and their elimination pathways.

Elimination Pathway	Commonly Administered Drugs in the ICU
CYP2C9/CYP2C19	Phenytoin Phenobarbital Clopidogrel Pantoprazole Warfarin Omeprazole Lansoprazole
CYP2D6	Metoprolol Propranolol Codeine Risperidone Tramadol
CYP3A	Midazolam Fentanyl Lidocaine Verapamil Diltiazem Amlodipine Amiodarone Nifedipine Alprazolam Morphine Carbamazepine Rifampin Diltiazem Dexamethasone Clarithromycin
CYP2B6	Propofol
CYP2A6	Dexmedetomidine
Phase II glucuronidation/UGT	Morphine
Renal Filtration	Gentamicin Sotalol
Renal Excretion	Atenolol Glycopeptides Aminoglycosides β-lactams Atracurium

Excreted Unchanged	Bretylium
N-acetyltransferase	Procainamide

Many of the drugs administered to critically ill patients have key pharmacokinetic properties such as large volumes of distributions, extensive binding to plasma proteins, and require hepatic metabolism as a primary mechanism of elimination. **Table 2** further details the pharmacokinetic characteristics of these commonly administered medications. To prevent ADEs, the Food and Drug Administration (FDA) and Center for Drug Evaluation and Research (CDER) have established *in vitro* and *in vivo* guidelines to test for metabolism-based and transporter-based drug-drug interactions (Huang, Temple *et al.* 2007, Services 2012). In contrast to these guidelines for new drugs that come to market, new pharmacological therapies are not upheld to the same detailed testing for potential drug-therapy interactions. Often times, a new therapy, such as therapeutic hypothermia, is implemented in the clinic with little to no knowledge of how it will impact drug pharmacokinetics. Without a better understanding of the potential alterations of a new pharmacological therapy, we put the patient at high risk for adverse drug events.

Table 2: Pharmacokinetic properties of commonly administered medications in the ICU.

	Primary Route of Elimination	Pathway(s) of Elimination	Volume of Distribution	Protein Binding	Half-life
ANALGESICS/SEDATIVE					
Fentanyl	Hepatic: 75%	CYP3A4	4 - 6 L/kg	80-85%	2.5-6.5 mins
Propofol	Hepatic: 90%	CYP2B6/UGT	60 L/kg	95-99%	30-60 mins
Dexmedetomidine	Hepatic: 95%	CYP450 & glucuronidation	118 - 152 L/kg	94%	2-2.67 hrs
Remifentanil	Hepatic: 90%		350 mL/kg	92%	3-10 mins
Midazolam	Hepatic: 63 - 80%	CYP3A4	1 - 3.1 L/kg	95%	1.8-6.4 hrs
Lorazepam	Hepatic: 88%	Conjugation	1.3 L/kg	91%	9-19 hrs
Ketamine	Hepatic	N-dealkylation, hydroxylation, conjugation, dehydration	2 - 3 L/kg	47%	2-3 hrs
Morphine	Hepatic: 90%	CYP2C, CYP3A; UGT	1 - 4.7 L/kg	30-40%	2-3 hrs
PARALYTICS					
Vecuronium	Hepatic: 15% Bile: 30 - 50%	CYP3A4 PGP Transporter	0.2 - 0.4 L/kg	30%	51-80 mins
Rocuronium	Bile: extensive Hepatic: 33%	CYP2D6 Renal	0.25 L/kg	30%	84-131 mins
Pancuronium	Hepatic: 57 - 69% Bile: 11%	Renal elimination & Bile	0.19 L/kg	77-91%	1.5-2.7 hrs
ANTI-ARRYTHMICS					
Lidocaine	Hepatic: 90%	CYP1A2, CYP3A	1.5 L/kg	60-80%	1.5–2.0 hrs
Amiodarone	Bile: primary	CYP2C9/19, CYP3A4	60 L/kg	33-65%	15-142 days
Digoxin	Hepatic: 57 – 80% Bile: 6 - 8%	glomerular filtration, PGP Transporter	4 - 7 L/kg	25%	36-48 hrs
Diltiazem	Hepatic: 35% Feces: 60-65%		3 - 13 L/kg	77-93%	3-6.6 hrs

ANTI-HYPERTENSIVE					
Verapamil	Hepatic: 70% Feces: 9 - 16%	CYP3A4, CYP2C9/19; PGP Transporter	3.8 L/kg	90%	3-7 hrs
Enalapril	Hepatic: 61%	OATP/MRP2 Transporters	?	50-60%	11 hrs
Metoprolol	Hepatic: 95%	CYP2D6 & CYP2C9	5.6 L/kg	15%	3-7 hrs
Valsartan	Feces: 83% Hepatic: 7-13%	OATP/MRP2 Transporters	17 L/kg	95%	6 hrs
Pressors and Iontropes					
Epinephrine	Hepatic	MAO/COMT	N/A	N/A	2 mins
Norepinephrine			N/A	N/A	
Phenylephrine	Hepatic: 80 - 86%	oxidative deamination; sulfation and some glucuronidation	40 L/kg	N/A	2-3 hrs
Milrinone	Hepatic: 80 - 85%	Active tubular secretion	0.3 - 0.47 L/kg	70%	1-3 hrs
Dopamine	Hepatic: 80%	MAO/COMT	1.8 - 2.5 L/kg		9 mins
Vasopressin	Hepatic: 5-10%		N/A	N/A	10-20 mins
ANTI-CONVULSANT					
Phenytoin	Bile: Extensive Renal	CYP2C9, CYP2C19; UGT	0.5 - 1.0 L/kg	90%	7-42 hrs
Phenobarbital	Hepatic	CYP2C9; hydroxylation and glucuronide conjugation; UGT		20-45%	2–7 days
Carbamazepine	Hepatic: 72% Feces: 28%	CYP3A4, CYP2C9; PGP/UGT Transporters	0.8 - 2 L/kg	76%	25-65 hrs
Keppra	Hepatic: 66%	enzymatic hydrolysis	0.7 L/kg	< 10%	6-8 hrs

ANTI-PLATELET/CLOTTING					
Warfarin	Hepatic: 92%	CYP2C9	0.14 L/kg	99.5%	20-60 hrs
Heparin	Hepatic		0.07 L/kg		1-2 hrs
Dalteparin	Hepatic: extensive		40 - 60 mL/kg	Low	3-5 hrs
Aspirin	Hepatic: 5.6 - 35.6%	hepatic conjugation	150 mL/kg	50-80%	15-20 mins 4.7-9 hrs
Clopidogrel	Hepatic: 50% Feces: 46%	CYP2C19		98%	6 hrs
Rivaroxaban	Hepatic: 66% Feces: 28%	CYP3A4/5 & CYP2J2	50 L/kg	92-95%	5-9 hrs
Dabigatran	Hepatic: 80%	esterases and glucuronidation	50-70 L/kg	35%	12-17 hrs
MISCELLANEOUS					
Quetiapine	Hepatic: 70 - 73%	CYP3A4	6 - 14 L/kg	83%	6 hrs
Haloperidol	Hepatic: 50-60% Feces: 15%	Glucuronidation; CYP3A4	9.5 - 21.7 L/kg	90%	18 hrs
Gentamicin	Hepatic: 70%	glomerular filtration	0.2 - 0.3 L/kg	<30%	1.5-3 hrs
Piperacillin / Tazobactam	Hepatic: 60-80%		0.18 - 0.3 L/kg	16%	36-80 mins
Vancomycin	Hepatic: 40 - 100%		0.2 - 1.25 L/kg	30-55%	
Pravastatin	Hepatic: 20% Feces: 37%		0.46 L/kg	43-55%	2.6-3.2 hrs
Pantoprazole	Hepatic: 71% Feces: 18%	CYP2C19/CYP3A4	11 - 24 L/kg	98%	1 hr
Famotidine	Hepatic: 25 - 70%	CYP450s	1 L/kg	15-20%	8-12 hrs
Corticosteroids	Hepatic	CYP3A4		Varies	Varies

Abbreviations: UGT = Uridine 5'-diphospho-glucuronosyltransferase; PGP = p-glycoprotein; MAO = Monoamine oxidases; COMT = Catechol-*O*-methyltransferase; OATP = organic anion-transporting polypeptide; MRP2 = Multidrug resistance-associated protein 2.

1.3 EFFECT OF THERAPEUTIC HYPOTHERMIA ON DRUG PHARMACOKINETICS

As previously discussed, therapeutic hypothermia has been shown to decrease the clearance of hepatically-metabolized drugs, which puts the patient at an elevated risk to reach super- or supra-therapeutic drug concentrations (Tortorici, Kochanek *et al.* 2007). With increased implementation comes a growing need to understand the ramifications of therapeutic hypothermia on other important factors of ICU care. One such factor is drug disposition and efficacy changes in the hypothermic patient. Specifically, clinical practitioners have postulated the question, "Should drug doses be altered during or after cooling in patients receiving therapeutic hypothermia?" The purpose of this section is to explore this question and present the current understanding of the effects of therapeutic hypothermia on the processes of absorption, distribution, metabolism and excretion, by providing specific evidence of drugs with altered and unaltered pharmacokinetics. Drugs are grouped by indication. **Table 3** summarizes the current pre-clinical and clinical studies investigating the effects of therapeutic hypothermia on drug pharmacokinetics.

Sedatives/Anesthetics

Midazolam.

Midazolam is part of the benzodiazepine class of drugs and is commonly administered as a sedative in the ICU. Hostler *et al.* randomized six healthy volunteers to receive 37°C or 4°C

saline infusion with or without magnesium. While the lowest temperature achieved in the hypothermic group was 35.4°C, pharmacometric analysis predicted a 11.1% decrease in midazolam clearance per each degree Celsius decrease in core body temperature in healthy volunteers (approximately a 44.4% decrease in clearance to a target clinical temperature of around 33°C) (Hostler, Zhou *et al.* 2010).

Bjelland *et al.* investigated the effects of hypothermia on the disposition of midazolam along with several other sedatives and anesthetics (fentanyl, morphine and propofol) in patients suffering from CA (Bjelland, Klepstad *et al.* 2013). A total of 15 CA patients who underwent hypothermic treatment to 33 – 34°C were enrolled in this case-control study and matched to critically ill patients in the ICU who did not receive hypothermia (n = 8). In contrast to Hostler *et al.*, the investigators found no difference in the volume of distribution (V_D), clearance (Cl), or elimination half-life ($t_{1/2}$) of midazolam between the hypothermic patients and the normothermic controls. This study involved a relatively small number of subjects with significant inter-individual variability in the midazolam time-concentration profiles in both groups. Further, the duration of hypothermia treatment across the 15 patients varied from 2 to 17 hours, which may have contributed to the large variability in the drug concentration profiles. In a follow-up observational study, Bjelland *et al.* investigated the effects of rewarming following hypothermia on the drug concentrations of midazolam in eight CA patients (Bjelland, Klepstad *et al.* 2014). In contrast to their previous study, they reported a 2.9% decrease in midazolam concentrations for every 1°C increase in temperature (11% total decrease from 33 to 37°C). One important difference in this study was the use of each patient as their own control which eliminated the high inter-individual variability seen in their previous study.

Bastiaans *et al.* reported no significant difference in the pharmacokinetics of midazolam between hypothermic patients (33°C) following resuscitation (n = 9) versus normothermic, non-resuscitated patients (n = 8) (Bastiaans, Swart *et al.* 2013). Population PK showed no different in the Cl or V_D of midazolam between the hypothermic and normothermic groups.

Another clinical study, by Welzing *et al.*, investigated the effect of hypothermia on midazolam PK in nine asphyxiated newborns treated with whole body hypothermia at 32 – 34°C for 72 hours (Welzing, Junghaenel *et al.* 2013). Neonates were administered a continuous infusion of midazolam (30 – 100 µg/kg/h). Population pharmacokinetic analysis was used to calculate the $t_{1/2}$, V_D, and Cl. In this small study, the authors report a midazolam Cl of 2.57 ml/kg/min and $t_{1/2}$ of 7.0 hours which was comparable to literature values in normothermic neonates. Interpretations of this study are limited by a small sample size with high inter-individual variability in midazolam metabolism, which makes it difficult to differentiate the effects of hypothermia versus other covariates, such as liver impairment, on the PK of midazolam.

Empey *et al.* demonstrated a 17.5% decrease in the systemic clearance of midazolam in hypothermic versus normothermic rats following CA (Empey, Miller *et al.* 2012). Following asphyxia, rats were cooled to 33°C or maintained under normothermic conditions at 37°C. Midazolam was administered via continuous infusion (1.5 mg/kg/h) and temperature was maintained over an eight-hour study duration to reach steady state. Significantly higher midazolam plasma concentrations were achieved in the hypothermic rats versus the normothermic rats. Noncompartmental analysis revealed that the increase in plasma concentrations was due to a 17.5% decrease in systemic clearance following CA.

Overall, the reported effect of TTM on the PK of midazolam varies from none to 11.1% decrease in systemic clearance per degree Celsius. Based on the preponderance of recent evidence, it is clear that the effect of TTM on midazolam PK is much less than that reported originally by Fukuoka *et al.* (Fukuoka, Aibiki *et al.* 2004). The difference between these studies may be attributed to small sample sizes (n = 8 – 15), different patient populations (neonates versus adults), different disease states (TBI, CA, healthy volunteers), varying duration of hypothermia (2 – 72 hours) and rewarming (8 hours up to 6 days) protocols, different PK models, and high inter-individual variability. Collectively, these results indicate that CYP3A4 metabolism is probably due to a combination of asphyxia and/or hypothermia with the hypothermia effect ranging between none to 11% per degree Celsius change.

Fentanyl

Fentanyl is an analgesic agent that is commonly administered in the ICU, and is often used during TTM. Fentanyl is extensively metabolized by CYP3A4. However, unlike midazolam, it is considered a high-clearance drug in humans. Based on the well-stirred model of hepatic drug clearance, the elimination of a high clearance drug such as fentanyl should be predominately affected by changes in hepatic blood flow (Pang and Rowland 1977).

Bjelland *et al.* reported a 45.5% decrease in the median total clearance of fentanyl was reported in 14 CA patients versus eight case-control matched critically-ill patients (36-38°C) [median [semi-interquartile range]: 726 [230] versus 1331 [678]; $P < 0.05$] (Bjelland, Klepstad *et al.* 2013). In a subsequent study, Bjelland *et al.* reported no change in fentanyl concentrations from the hypothermia to rewarming phases (Bjelland, Klepstad *et al.* 2014). The lack of change in fentanyl concentrations during rewarming, despite significant changes during hypothermia,

may be attributed to the long half-life of fentanyl relative to the short duration of rewarming. Since hepatic blood flow was not able to be measured in either of these clinical studies, it is difficult to mechanistically evaluate how temperature changes affected liver blood flow and contributed to overall changes in fentanyl PK. In conclusion, these recent studies are consistent with previous findings in which reduced CYP3A activity decreased fentanyl metabolism with potentially undefined contribution of hepatic blood flow.

In the same pre-clinical study in which Empey *et al.* reported a decrease in midazolam clearance, they also reported a 20.5% decrease in the systemic clearance of fentanyl in hypothermic versus normothermic rats following CA (Empey, Miller *et al.* 2012). Enzyme kinetics revealed that the decrease in formation of the Cyp3a-dependent metabolite, norfentanyl, was attributed to an overall decrease in maximal velocity, V_{max}, and not due to a change in enzyme affinity, K_m.

Phenobarbital.

Phenobarbital (PB) is a barbiturate indicated for sedation and epilepsy. Phenobarbital is primarily metabolized in the liver by the cytochrome P450 2C19 (CYP2C19) isoform. Three clinical studies have investigated the effects of hypothermia on PB PK in neonates, a population where PB is commonly used as a first-line agent to prevent neonatal seizures (Filippi, la Marca *et al.* 2011, van den Broek, Groenendaal *et al.* 2012, Shellhaas, Ng *et al.* 2013).

Filippi *et al.* investigated PB PK at two different doses in 19 asphyxiated newborns who were undergoing 72 hours of mild hypothermia to 33.5°C (Filippi, la Marca *et al.* 2011). Noncompartmental analysis of steady-state PB concentrations demonstrated a higher C_{avg}, C_{min}, and C_{max} in the hypothermic neonates as compared to literature values reported for normothermic

neonates. Further, the $t_{1/2}$ of PB was 32.1% longer in hypothermic neonates than the average literature values in normothermic neonates ($t_{1/2}$ = 173.9 ± 62.5 h versus 114-118 h). Additionally, the $Versus_s$ was also higher than reported values and the Cl was on the lower end of reported values in normothermic neonates. The authors conclude that hypothermia is likely mediating the decrease in PB elimination but acknowledge that asphyxia may also be contributing to PK changes.

In contrast to Filippi *et al.*, a clinical study performed by van den Broek *et al.* reported no effect of hypothermia (33.5°C) for 72 hours on PB PK in asphyxiated neonates (van den Broek, Groenendaal *et al.* 2012). This retrospective study identified 31 neonates with HIE who were administered an intravenous loading dose of PB of 20 mg/kg with additional doses administered as needed in subsequent days. Phenobarbital concentration was measured in a total of 87 plasma samples obtained from the hypothermic phase (*n* = 69) and rewarming and pre-hypothermic phases (*n* = 18). Population PK analysis showed that temperature did not have a clinically significant effect on the Cl or V_D of PB. Interestingly, van den Broek *et al.* noted that the clearance of PB in the asphyxiated neonates was reduced compared to those values reported in non-asphyxiated neonates. This was consistent with findings by Gal *et al.* who reported a reduction in the clearance of asphyxia neonates by over half compared to non-asphyxiated neonates (Gal, Toback *et al.* 1984). Van den Broek *et al.* postulated that the effect of asphyxia could be predominately driving changes in the PB clearance, and the effect of temperature may not have an additional contributing effect. Since the effect of asphyxia on PB PK was not tested by Filippi *et al.*, this could be the main covariate contributing to the discrepancies in conclusions between these two studies.

Shellhaas *et al.* also found no effect of TTM on the clearance of PB in neonates with HIE (Shellhaas, Ng *et al.* 2013). This retrospective study included 20 neonates with HIE who were administered PB and TTM (33.0 – 35.0°C), and 19 neonates with HIE who were administered PB but did not undergo TTM. Using a population PK approach, they also found no effect of temperature on the Cl or V_D of PB between the hypothermic and normothermic neonates. Due to the retrospective nature of this study, exact body temperatures and times were not available to include in analysis and therefore the authors were unable to compare the exact relationship between dose, PB PK, and body temperature. Instead, the subjects could only be grouped based on hypothermic or normothermic classification.

Collectively, these studies indicate that the PK of PB may be altered in neonates with HIE who are undergoing TTM. However, the driving force behind that change in PK, and the interplay of disease versus temperature, remains to be determined. It appears that the initial findings reported by Filippi *et al.* which attribute temperature to changes in PB PK may be confounded by the effect of asphyxia. More recent population PK studies have failed to show an effect of temperature on PB clearance or volume of distribution. It is likely that any effect of hypothermia on PB PK is due to a decrease in CYP2C9 activity, however the effect of asphyxia on PB PK is well known and any additional contributions due to a temperature-dependent change in CYP2C9 activity seems unlikely. Further, these studies provide evidence for neonates with HIE only, and whether these results extrapolate to older populations in children and adults is still unknown.

Propofol.

Propofol is a sedative and analgesic agent commonly used in neurocritical care in the adult ICU. Propofol is predominately metabolized by CYP2B6 with a small amount undergoing glucuronidation by UDP-glucuronosyltransferase 1A9. Two recent clinical studies have investigated hypothermia on propofol PK. In the same study that investigated midazolam PK by Bjelland *et al.,* the authors also investigated propofol PK in 14 hypothermic patients suffering from CA (Bjelland, Klepstad *et al.* 2013). Hypothermic patients had a 23.2% lower clearance of propofol than normothermic patients [median (semi-interquartile range): 2046 (305) versus 2665 (223) ml/min; p-value = 0.035]. In a subsequent study, Bjelland *et al.* investigated the concentrations of propofol during rewarming in 14 CA patients (Bjelland, Klepstad *et al.* 2014). The concentration of propofol decreased 3.1% per degree Celsius increase in body temperature (approximately 12.4% total change in concentration from 33 – 37°C) during rewarming. However, no PK parameters were reported in this study. Propofol, like fentanyl, is considered a high clearance drug and since hepatic blood flow was not measured in either of these two clinical studies, it is difficult to identify the mechanism underlying the reductions in Cl. Collectively, these two studies indicate hypothermia may decrease propofol clearance due to a combined effect of hepatic blood flow and/or CYP2B6 activity leading to an increase in concentrations.

Dexmedetomidine.

Dexmedetomidine is a sedative agent which has also been shown to exhibit anti-shivering properties. In addition, dexmedetomidine has been associated with a lower rate of delirium development as compared to other sedatives in ICU patients (Li, Yang *et al.* 2017). Dexmedetomidine undergoes extensive hepatic metabolism primarily via CYP2A6 isoform and

direct glucuronidation. To date, one pre-clinical study in piglets has investigated the effect of TTM on the PK of dexmedetomidine (Ezzati, Broad *et al.* 2014). Nine piglets underwent cerebral hypoxia ischemia followed by whole-body hypothermia to 33.5°C for 72 hours. Population PK analysis revealed a 32.7% decrease in dexmedetomidine clearance in hypothermic piglets following hypoxia-ischemia. A limitation of this study is that it included only injured piglets undergoing TTM and therefore they could not separate out the effects of injury versus temperature on the PK changes of dexmedetomidine. Overall, the decrease in clearance reported in this study was attributed to combined effects of injury and cooling. Future clinical studies with larger sample size will be needed to determine the contribution of injury and temperature on the PK of dexmedetomidine, particularly given the recent consideration and evaluation of dexmedetomodine as an anti-shivering agent and reduced rates of ICU delirium (Doufas, Lin *et al.* 2003, Callaway, Elmer *et al.* 2015).

Morphine.

Morphine is an analgesic that is commonly administered in the ICU. In contrast to many of the drugs discussed so far which undergo Phase I CYP450 metabolism, morphine undergoes Phase II glucuronidation. Two clinical studies have demonstrated an effect of hypothermia on morphine concentrations and PK (Roka, Melinda *et al.* 2008, Bjelland, Klepstad *et al.* 2013). Roka *et al.* found significantly higher morphine concentrations in hypothermic neonates with HIE as compared to normothermic neonates with HIE (Roka, Melinda *et al.* 2008). In this observational study, 10 neonates with HIE underwent hypothermic treatment to 33 – 34°C and 6 neonates with HIE were maintained normothermic. Mean morphine concentrations after 72 hours of cooling were 40.5% higher in the hypothermic group than in the normothermic group (373 ±

125 ng/mL versus 222 ± 73 ng/mL). Further, the hypothermic group showed a trend in higher morphine AUC than in the normothermic group despite no difference in the morphine infusion rates between groups (18608 ± 8384 ng/h/mL versus 12135 ± 3481 ng/h/mL; P = 0.051). The median morphine clearance was estimated from a subset of patients with samples available at each time point (0.69 mL/min/kg versus 0.89 mL/min/kg) however, the hypothermic group never reached steady-state preventing the calculation of a steady-state morphine clearance in this group.

Bjelland *et al.* reported the median half-life of morphine was 36.8% higher in ICU patients undergoing hypothermia versus the normothermia group (median (semi-interquartile range); 266 (43) min versus 168 (11) min) (Bjelland, Klepstad *et al.* 2013). Further the median total CL decreased by 28.8% in the hypothermic versus the normothermic group (median (semi-interquartile range); 1201 (283) ml/min versus 1687 (200) ml/min). No significant difference in apparent volume of distribution was seen between groups (median (semi-interquartile range); 413 (89) l versus 435 (28) l). Collectively, these two clinical studies indicate that hypothermia decreases morphine clearance, which is likely due to a decrease in the Phase II metabolic glucuronidation pathway. However as was seen in many of the previous studies, the change in drug PK is most likely a combined effect of injury and temperature. The degree to which each of these factors contributes to changes in drug PK most likely varies across drugs.

Anticonvulsants

Phenytoin

Phenytoin is a commonly administered anticonvulsant, which primarily undergoes hepatic metabolism via CYP2C9 and CYP2C19. The unique PK of phenytoin include a relatively

long half-life and saturable metabolism within the typical therapeutic range. We conducted a clinical study investigating the effects of hypothermia on phenytoin PK in children following TBI (Empey, de Mendizabal *et al.* 2013). Nineteen children with TBI in a prospective RCT were randomized to receive hypothermia or normothermia treatment. Using a population PK approach, hypothermia was shown to reduce phenytoin elimination, similar to the Iida *et al.* study. Specifically, hypothermia led to an overall decrease of approximately 50% in the V_{max} of phenytoin. Importantly, these supra-therapeutic phenytoin concentrations remained elevated days after the end of cooling and rewarming and into the post-treatment period. This was a similar finding to Bjelland *et al.* who reported temperature effects on fentanyl PK long after the hypothermia phase. Collectively, these results are an important finding with regard to pharmacotherapy and TTM, namely, that drugs with long half-lives, such as phenytoin and fentanyl, when administered to patients undergoing TTM, should continue to be monitored even into the post-treatment period and subsequent dose-adjustments should be made accordingly. This is particularly true given that rewarming has been identified as a period of potential heightened hemodynamic and brain instability in brain injured patients (Hutchison, Ward *et al.* 2008, Abend, Topjian *et al.* 2009). Furthermore, this issue is particularly important with sedatives such as fentanyl because this drug can mask brain activity during end-of-life assessment and withdrawal of support decision making after the rewarming period.

Lidocaine.

Lidocaine is an antiarrhythmic agent and is also used as a second or third-line treatment for neonatal seizures. Lidocaine undergoes hepatic metabolism via CYP1A2, and is classified as a high clearance drug therefore its hepatic clearance is predominately impacted by changes in

hepatic blood flow. Van den Broek *et al.* investigated the effects of TTM on the PK and efficacy of lidocaine in neonates with encephalopathy (van den Broek, Rademaker *et al.* 2013). A total of 22 asphyxiated neonates underwent hypothermia to 33.5°C for 72 hours. Population PK analysis revealed a 24% decrease in lidocaine clearance in the hypothermic versus normothermic neonates. The reduction in clearance was attributed to a decrease in hepatic blood flow, which is a known physiologic effect of hypothermia. In conclusion, the authors recommend using the same dose of lidocaine with a slight decrease in the loading infusion duration by 30 minutes.

Antimicrobials

Gentamicin

Gentamicin is an antimicrobial agent that is administered to treat infections in critically ill patients. Gentamicin is primarily renally-eliminated in an unchanged form via glomerular filtration. Since gentamicin is known to be nephrotoxic, blood concentrations are routinely measured in the ICU.

Several clinical studies have investigated the effect of TTM on gentamicin PK in neonates (Liu, Borooah *et al.* 2009, Frymoyer, Meng *et al.* 2013, Mark, Solomon *et al.* 2013, Ting, Kwan *et al.* 2014). Liu *et al.* was the first to report no significant effect of TTM on the serum gentamicin concentration in neonates with HIE (Liu, Borooah *et al.* 2009). Trough serum gentamicin concentrations were not different between the hypothermic and normothermic groups (2.19 ± 1.7 mg/L hypothermic versus 2.30 ± 2.0 mg/L normothermic). However, neonates in both groups had elevated serum concentrations, which can also be explained by the strong correlation between acute kidney dysfunction and elevated gentamicin concentrations. Similar to

previous studies, this suggests a potential effect of disease over temperature on the PK of gentamicin. However, no PK parameters were reported in this study.

Mark *et al.* also investigated the effects of hypothermia on gentamicin PK in neonates with HIE (Mark, Solomon *et al.* 2013). In this retrospective case-control study, 16 neonates with HIE who underwent hypothermia (33.5°C for 72 hours) and received at least 2 doses of gentamicin were included. Neonates with HIE (n = 7) who did not undergo hypothermia treatment but received at least two doses of gentamicin were included in the control group. The hypothermic neonates had a 40% increase in half-life (9.16 ± 2.08 hours versus 6.56 ± 1.81 hours; P<0.01), a 25.5% decrease in Cl (0.04 ± 0.01 L/kg.h-1 versus 0.05 ± 0.01 L/kg.h-1; P<0.01), and a 28% decrease in the elimination rate constant k_e (0.08 ± 0.02/h versus 0.11 ± 0.03/h; P<0.01). The hypothermic neonates also had an average trough gentamicin concentration 2.2 times higher than the normothermic neonates (1.68 ± 0.69 µg/mL versus 0.77 ± 0.53 µg/mL; P<0.01). These findings are consistent with those reported in our previous review by Koren *et al.* in which the half-life of gentamicin was increased by 39% in hypothermic pigs and the k_{el} and Cl were decreased by 27% and 51%, respectively (Koren, Barker *et al.* 1985). Both the hypothermic study in pigs by Koren *et al.* and the clinical study by Mark *et al.* demonstrate a combined effect of injury and temperature on the pharmacokinetics of gentamicin.

Frymoyer *et al.* further investigated the effect of hypothermia on gentamicin PK in neonates with HIE (Frymoyer, Meng *et al.* 2013). A retrospective chart review identified 29 neonates with HIE (47 gentamicin concentrations) who underwent hypothermia and received an iv gentamicin dose of 5 mg/kg Q 24 hours. A population PK analysis was performed to determine the Cl and V_D of gentamicin in this patient population and subsequent simulations were done to determine a dosing regimen that would achieve therapeutic concentrations.

Clearance in this patient population was 0.118 L/h, which was 25 – 50% lower than reported literature values in normothermic neonates with HIE. In contrast, the V_D in the hypothermic neonates was similar to reported values in normothermic neonates. Simulations based off of these PK parameters predict a dosing regimen of gentamicin every 36 hours, instead of the typical 24 hours, to achieve concentrations below the recommended 2 mg/L.

Finally, Ting *et al.* also investigated the effect of TTM on gentamicin PK in neonates with moderate to severe HIE (Ting, Kwan *et al.* 2014). They identified 15 neonates with HIE who underwent hypothermia and 19 neonates with HIE maintained normothermic. The hypothermic group had a 26.8% longer half-life than the normothermic group (9.57 hours versus 7.01 hours; P = 0.007) and a high number of neonates in the hypothermic group had elevated gentamicin concentrations compared to those in the normothermic group.

The majority of these studies indicate that gentamicin clearance is decreased in neonates with HIE treated with hypothermia. The results suggest that a decrease in the dose of gentamicin or an increase in the dosing interval may be beneficial when gentamicin is being administered during hypothermia. However, all of these studies have significant limitations: they are retrospective analyses with a relatively small sample size and are mostly in neonates and have a limited number of plasma samples based on what was previously recorded. Further, these studies cannot separate the combined effect of injury and hypothermia on the PK of gentamicin.

1.3.1 Effect of Therapeutic Hypothermia on Drug Transport

In addition to drug metabolism, drug transporters also play a key role in determining a drug's PK parameters. P-gp is expressed in tissues such as the liver, kidneys, intestines, and at the blood-brain barrier and is known to transport a number of clinically important drugs. Although less is

known about the effect of hypothermia on the transport of drugs, it is reasonable to postulate that active drug transport would be decreased similarly to active drug metabolism, since transporter proteins are temperature sensitive and would undergo a similar inactive response to cooling. Jin *et al.* investigated the effect of targeted temperature management on the P-glycoprotein (P-gp) drug transporter at 37, 32, 30, 25, and 4°C in an *in vitro* cell culture study (Jin, Sakaeda *et al.* 2006). Radiolabeled probe drugs were used to quantify the overall flux (transport) across the cell monolayers of P-gp-overexpressing cells. The active transport of [^{3}H]digoxin was decreased by ~50% from 37 to 32°C whereas no change in the transport of the paracellular marker, [^{14}C]inulin, was seen indicating a decrease in the active drug transport of P-gp. These findings are consistent with a previous pre-clinical study which suggests that hypothermia decreases the active processes of renal tubular secretion but has no effect on the passive process of renal filtration (Nishida, Okazaki *et al.* 2007). Future studies are still warranted that quantify the absolute change in drug transporter activity and delineate how these changes may contribute to overall drug PK.

1.3.2 Interplay of Therapeutic Hypothermia and Injury on Drug Pharmacokinetics

Based on recent studies, it is apparent that changes in drug PK are due to a combination of cardiac arrest and hypothermia-mediated effects, and considerations for drugs which undergo CYP450 elimination pathways should take both into consideration. Previously our laboratory investigated the interplay of therapeutic hypothermia and cardiac arrest on CYP450 pathways in rats (Zhou, Empey *et al.* 2011). In addition to its sedative use in the ICU, midazolam is also an established cytochrome-P450 3A (CYP3A) probe (Yuan, Madani *et al.* 2002, Eap, Bouchoux *et al.* 2004). Midazolam was administered with diclofenac, chlorzoxazone, and dextromethorphan

to probe CYP3A, CYP2C, CYP and CYP2D activity respectively. Midazolam clearance decreased in the CA hypothermia rats versus the normothermic shams (681.6 ± 190.0 versus 1268.8 ± 348.9 ml/kg/h), which indicates a combined effect of injury and hypothermia. Similarly, CZN clearance decreased in a hypothermic rat model of CA as compared to sham normothermic rats (229.6 ± 75.6 versus 561.89 ± 215.9 ml/kg/h; $p < 0.05$). In contrast to chlorzoxazone and midazolam, the clearance of diclofenac or dextromethorphan was not significantly different between any of the groups. Two important findings from this study are that 1) the CA and hypothermia-mediated changes are specific to CYP450 isoform and 2) ischemic injury is a significant contributor to reduced metabolism in this injury model producing decreased clearance of chlorzoxazone and midazolam. Future studies should investigate the extent of which injury versus cooling effect drug PK in clinical studies as well as the interplay of these effects on drug pharmacodynamics.

1.4 EFFECT OF THERAPEUTIC HYPOTHERMIA ON DRUG PHARMACODYNAMICS

In addition to an effect on a drug's pharmacokinetics, therapeutic hypothermia can also alter a drug's pharmacodynamics properties. More recently, the effects of hypothermia on the pharmacodynamics of anti-platelet agents has been investigated.

Anti-platelets.

Clopidogrel is a pro-drug that is used to prevent platelet aggregation. Unlike the majority of drugs administered in the ICU, clopidogrel is administered orally and depends on metabolic

activation by CYP2C19 as well as other CYP450 enzymes. Bjelland *et al.* investigated the effect of hypothermia on the clopidogrel's efficacy to inhibit platelets (Bjelland, Hjertner *et al.* 2010). In this prospective study, 25 CA patients received hypothermia (33 - 34°C) for 24 hours. Patients received a loading dose of clopidogrel of 300 mg orally followed by maintenance doses of 75 mg. Whole blood samples were collected on day 1 (n = 25) during the cooling phase and on day 3 (n = 16). During hypothermia, the number of blood samples that had a satisfactory effect of clopidogrel was 0/25. One possible explanation may be a decreased metabolic conversion of clopidogrel to its active form during cooling, however lack of a PK analysis in this study does not rule out this possibility from other potential pharmacodynamics effects such as genetic variants or function of the gastrointestinal tract.

An *in vitro* study by Ferreiro *et al.* investigated the effect of hypothermia on the pharmacodynamics of clopidogrel and aspirin (Ferreiro, Sanchez-Salado *et al.* 2014). In this study blood samples were obtained from 20 patients who underwent percutaneous coronary intervention and received loading doses of aspirin and clopidogrel. Mild hypothermia to 33°C led to a decrease in clopidogrel-mediated platelet inhibition while having no effect on the pharmacodynamics response of aspirin.

More recently Ibrahim *et al.* investigated the effect of hypothermia on the pharmacodynamics of three anti-platelet agents clopidogrel, prasugrel, and ticagrelor in patients with acute coronary syndrome (Ibrahim, Christoph *et al.* 2014). In this study, they demonstrated that the platelet inhibiting effect of all three drugs was significantly decreased under hypothermic conditions. Clopidogrel showed the largest reduction in platelet inhibition in hypothermic versus normothermic groups (hypo versus normo: 66.39% ± 19.1 versus 33.36% ± 22.1; p-value < 0.001). This study concluded that CA patients who are undergoing hypothermia versus

normothermia have increased rates of non-responders to P2Y12 receptor inhibitors such as clopidogrel, prasugrel, and ticagrelor.

Current evidence indicates that mild hypothermia to 33°C leads to a pharmacodynamic change in drug-mediated platelet response. Specifically, the platelet inhibitory effect of the P2Y12 receptor inhibitors clopidogrel, prasugrel, and ticagrelor is decreased, with clopidogrel showing the most marked change in pharmacodynamics response. However, since clopidogrel requires conversion into its active metabolite by CYP3A and CYP2C9, the increase in clopidogrel non-response may be due to a combination of a decrease in metabolic conversion to active drug (PK change) as well as a pharmacodynamics effect. More studies are needed to understand how the effect of hypothermia on platelet activation versus drug metabolism is dictating this change in response.

Table 3: Effect of hypothermia on drug pharmacokinetics and response in pre-clinical and clinical studies.

Drug	**Disease/Model**	**Hypothermia Protocol**	**Hypothermia PK Effects (versus Normothermia)**	**Reference** [Ŧ]
Clinical Studies				
Midazolam	Healthy volunteers	35.4°C 3 hours	↓ Cl	(Hostler, Zhou *et al.* 2010)
Morphine Midazolam Fentanyl Propofol	CA Patients	36 – 38°C, 33 - 34°C 12 – 24 hours	↑ $t_{1/2}$ and ↓ Cl of morphine, ↔ V_d ↔ Cl midazolam, ↔ V_d, ↔ $t_{1/2}$ ↓ Cl_{tot} of fentanyl ↓ Cl_{tot} of propofol	(Bjelland, Klepstad *et al.* 2013)
Remifentanyl Propofol Fentanyl Midazolam	CA patients	33 - 34°C 24 hours	↓ concs of remifentanyl, propofol, and midazolam during rewarming ↔ conc of fentanyl	(Bjelland, Klepstad *et al.* 2014)
Midazolam	Resuscitated patients	33°C 24 hours	↔ Cl, V_1, V_2, or Q	(Bastiaans, Swart *et al.* 2013)
Midazolam	Asphyxiated Neonates	32 – 34°C 72 hours	↔ Cl	(Welzing, Junghaenel *et al.* 2013)
Phenobarbital	Neonates with HIE	33.5°C 72 hours	↑ plasma concs, ↑ $t_{1/2}$	(Filippi, la Marca *et al.* 2011)
Phenobarbital	asphyxiated neonates	33.5°C 72 hours	↔ Cl, ↔ V_d	(van den Broek, Groenendaal *et al.* 2012)

Phenobarbital	Neonates with HIE	36 – 38°C, 33 – 35°C 72 hours	↔ Cl, ↔ V_d	(Shellhaas, Ng *et al.* 2013)
Phenytoin	Children with severe TBI	36.5 – 37.9°C, 32 – 33°C 48 hours	↓ V_{max}, ↔ K_m	(Empey, de Mendizabal *et al.* 2013)
Lidocaine	Asphyxiated neonates	33.5°C 72 hours	↓ Cl	(van den Broek, Rademaker *et al.* 2013)
Gentamicin	Neonates with encephalopathy	37°C, 33 – 34.5 °C 72 hours	↔ serum concs, ↔ Cl	(Liu, Borooah *et al.* 2009)
Gentamicin	Neonates with HIE	33.5°C 72 hours	↑ trough serum cons, ↓k_e, ↑ $t_{1/2}$, ↓ Cl, ↔ V_d	(Mark, Solomon *et al.* 2013)
Gentamicin	Neonates with HIE	33.5°C 72 hours	↓ Cl, ↔ V_d compared to previous reports	(Frymoyer, Meng *et al.* 2013)
Gentamicin	Neonates with HIE	37°C, 33.5°C 72 hours	↑ $t_{1/2}$, ↓k_e, ↓ Cl, ↑ C_{max} and C_{min}, ↑ AUC	(Ting, Kwan *et al.* 2014)
Morphine	Neonates with HIE	33 – 34°C 72 hours	↑ serum concs, ↑ AUC, ↓ Cl	(Roka, Melinda *et al.* 2008)
Clopidogrel	CA patients	33 – 34°C 24 hours	↓ clopidogrel-mediated platelet inhibition	(Bjelland, Hjertner *et al.* 2010)

Clopidogrel Prasugrel Ticagrelor	CA patients	32 – 34°C 12-24 hours	↓ clopidogrel-mediated platelet inhibition ↓ prasugrel-mediated platelet inhibition ↓ ticagrelor-mediated platelet inhibition	(Ibrahim, Christoph *et al.* 2014)
Pre-Clinical Studies				
Midazolam Fentanyl	CA rats	32.5 – 33.5°C 8 – 10 hours	↑ plasma concs of midazolam and fentanyl ↓ Cl_s of midazolam and fentanyl ↔ brain to plasma ratio	(Empey, Miller *et al.* 2012)
Dexmedetomidine	Piglets with HIE	33.5°C 18 – 24 hours	↑ plasma concs, ↓ Cl	(Ezzati, Broad *et al.* 2014)
Midazolam Diclofenac Dextromethorphan Chlorzoxazone	CA Rats	37°C, 32.5 – 33°C 8 hours	↓ Cl of midazolam and chlorzoxazone ↔ Cl of diclofenac or dextromethorphan ↓ V_1 of midazolam and dextromethorphase and V_2 for chlorzoxazone	(Zhou, Empey *et al.* 2011)

Phenolsulfonphthalein (PSP) Indocyanine green (ICG) Fluorescein isothiocyanate-dextran (FD-4)	Rats	37°C, 32°C, or 28°C 5 hours	↑PSP plasma concs; ↓ PSP Cl ↑ ICG plasma concs; ↓ ICG Cl ↔ FD-4 Cl	(Nishida, Okazaki *et al.* 2007)
Digoxin Inulin	*In vitro* cell culture	37°C, 32°C, 30°C, 25°C, and 4°C	↓ activity of ABCB1 drug transporter ↔ in paracellular transport	(Jin, Sakaeda *et al.* 2006)
Clopidogrel Aspirin	*In vitro* blood samples from patients with myocardial infarction	37°C, 33°C	↓ clopidogrel-mediated platelet inhibition ↔ aspirin-mediated platelet inhibition	(Ferreiro, Sanchez-Salado *et al.* 2014)

[ǂ] Pre-clinical and clinical studies ranging from July 2006 – October 2015

1.5 CONCLUSIONS

This chapter has demonstrated that therapeutic hypothermia significantly alters drug disposition including drug metabolism and drug response during each of the phases of cooling, re-warming, and post-treatment. Specifically, the studies outlined in this Chapter indicate that during cooling, drug metabolism is significantly decreased. This may lead to the conclusion that the dose of certain drugs should be reduced in order to avoid toxicity. However, the effects of hypothermia on drug response are variable and less predictable. Depending on drug, a reduction in potency or no change in potency has been shown. Overall, the effects of hypothermia during cooling suggest a narrowing of the therapeutic index of multiple drugs used in the ICU.

More recently, clinical studies have shown that drug concentrations continue to remain elevated after re-warming. Depending on the half-life of the drug, this effect can be seen for several days into the post-treatment phase. Due to the range of time dependent effects, it is essential that clinicians fully appreciate the potential changes in drug disposition during and after cooling. In some cases, this may require monitoring drug levels carefully during and after therapeutic hypothermia treatment. Further, the contribution of injury-mediated versus hypothermia-mediated effects on drug disposition will be an important area of research to help tailor dosing guidelines across different patient populations.

As we further evaluate the interplay of hypothermia-mediated effects on drug disposition new opportunities will arise for future research to systematically evaluate these effects. Additional clinical trials are needed to investigate drugs which are commonly administered during therapeutic hypothermia and in the ICU but have not been studied yet. Furthermore,

therapeutic hypothermia continues to be evaluated in new disease states, such as stroke and epilepsy. As this research progresses, it will also be important to explore the pharmacokinetics of the drugs used during these studies. This will serve two primary purposes. First, it will determine how to safely and effectively administer drugs in these patient populations under these hypothermic conditions. Second, it will distinguish any potential adverse drug events from hypothermia treatment to determine if certain drugs are beneficial or harmful during hypothermia.

The clinical studies outlined throughout this chapter can provide us with a theoretical time-course of hypothermia-mediated alterations on drug pharmacokinetics. As previously demonstrated, therapeutic hypothermia produces a significant decrease in drug metabolism during the period of cooling. Recent evidence has shown that this decrease in metabolism continues longer than initially projected. Hypothermia-mediated effects on PK can be seen into the rewarming phase and continue into the post-treatment phase even when core temperature is returned to normal body temperature. Future studies are needed in order to provide specific dosing recommendations for medications administered during each phase of treatment: hypothermia, rewarming, and post-treatment. In conclusion, research has shown that clinically significant hypothermia-drug interactions are a factor to be considered in the therapeutic course of these patients.

The work presented throughout this dissertation aims to evaluate the effects of therapeutic hypothermia on drug pharmacokinetics, specifically focusing on metabolism and drug transport in pre-clinical and clinical studies. As discussed throughout this Chapter, hypothermia can affect metabolism, absorption, elimination, distribution and response of many drugs. We have chosen to focus this work on the effects of cooling on the CYP450-metabolism

of phenytoin and the ABC drug transporter pathway system. Many of the previous clinical studies have demonstrated that hypothermic temperatures affect drugs metabolized by CYP450's during cooling. However, there are few robust pharmacokinetic studies to elucidate the mechanisms behind these metabolic-changes. The majority of studies describe a change in drug levels and a change in drug clearance. By conducting a robust pharmacokinetic analysis we are able to better describe the causes behind these changes. In addition we have chosen to investigate the effects of cooling on drug transport since little is known about how this important component of drug disposition is affected during cooling.

1.6 HYPOTHESIS

We aim to determine the effect of therapeutic hypothermia on drug disposition by expanding what is currently known on drug metabolism and conducting the first studies to investigate drug transport. We hypothesized that therapeutic hypothermia will lead to a decrease in phenytoin elimination due to a decrease in CYP450 metabolism in pediatrics following a cardiac arrest. We further hypothesized that therapeutic hypothermia will decrease the active drug transport of three ABC transporters (ABCB1, ABCG2, and ABCC1) but have no effect on passive transport across cell membranes. These studies will be the first to investigate the effects of therapeutic hypothermia on three ABC drug transporters. In order to evaluate these hypotheses the following specific aims have been proposed by chapter.

Specific Aim 1: Develop a method to quantify concentrations of phenytoin and levetiracetam in small volumes of pediatric serum samples.

Specific Aim 2: Determine the effects of therapeutic hypothermia, and other clinical covariates, on the pharmacokinetics of phenytoin in pediatrics following cardiac arrest using a population pharmacokinetic approach.

Specific Aim 3: Evaluate the effects of therapeutic hypothermia on the ABC drug transporter activity of ABCB1, ABCG2, and ABCC1 by conducting permeability flux assays using an *in vitro* cell culture model.

2.0 ANALYTICAL METHOD DEVELOPMENT OF PHENYTOIN AND LEVETIRACETAM

[Anderson KB, Liput SL, Fink E, Kochanek PM, Empey PE, Poloyac SM. A rapid UPLC-MS/MS method for the simultaneous measurement of phenytoin and levetiracetam in small volume pediatric serum samples. Trends in Chromatography 2016, In Submission]

2.1 INTRODUCTION

Following a cardiac arrest, pediatric patients are at high risk to develop seizures as a result of hypoxic-ischemic insult (Nishisaki, Sullivan *et al.* 2007, Glass, Glidden *et al.* 2009, Binks and Nolan 2010, Tress, Kochanek *et al.* 2010). For this reason, children may receive anti-epileptic drugs (AED) prophylactically to prevent the onset of CA-induced seizures or to treat actual seizures. Multiple factors can affect drug metabolism in critically ill patients including organ dysfunction following cardiac arrest (McKindley, Hanes *et al.* 1998, Power, Forbes *et al.* 1998, Haas and Forrest 2006, Roberts, Kilgannon *et al.* 2013, Tujjar, Mineo *et al.* 2015), targeted temperature management (TTM) (Tortorici, Kochanek *et al.* 2007, Empey, Velez de Mendizabal *et al.* 2013), inflammation (Vet, de Hoog *et al.* 2011, Vet, de Hoog *et al.* 2012), and co-administered medications (Smithburger, Kane-Gill *et al.* 2012).

Previously our laboratory demonstrated an important need to monitor AEDs in children with traumatic brain injury particularly during the hypothermic and re-warming phases of TTM (Empey, Velez de Mendizabal *et al.* 2013). Specifically, phenytoin levels reached supra-therapeutic concentrations both during and following hypothermic treatment due to a decrease in drug metabolism. The methods and results of this PK study will be further discussed in Chapter 3. It is currently unknown how pharmacokinetics of AEDs are affected following pediatric CA. To address this, we aimed to develop an assay to simultaneously quantify two commonly administered AEDs, phenytoin (PHY) and levetiracetam (LEV), in the serum of pediatrics following CA.

To date, several methods have been developed to quantify LEV (Pucci, Bugamelli *et al.* 2004, Rao, Ravi *et al.* 2004, Martens-Lobenhoffer and Bode-Boger 2005) or PHY (Haroon and Keith 1983, Kouno, Ishikura *et al.* 1993, Cwik, Liang *et al.* 1997) in human plasma using high-performance liquid chromatography (HPLC). However, the majority of these methods require long run times, lack sensitivity and require large sample volumes (0.5 – 1.0 mL) which often cannot be obtained from children. More recently, ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS/MS) has been used to quantify drug levels in plasma due to its improved resolution and increased sensitivity from conventional methods. However, few methods have been developed to quantify AEDs using UPLC-MS/MS (Blonk, van der Nagel *et al.* 2010, Shibata, Hashi *et al.* 2012, Karinen, Vindenes *et al.* 2015) and no studies report the simultaneous quantification of PHY and LEV in small serum volumes. Therefore, we aimed to develop a UPLC-MS/MS assay to quantify LEV and PHY in pediatric serum volumes of 20 μL.

In the current study, we established a fully validated, sensitive, and accurate UPLC-MS/MS method for the simultaneous quantification of LEV and PHY. This method is the first to report a simple one-step sample preparation method to quantify LEV and PHY in a serum sample volume of 20 μL. This assay was used to quantify all pediatric serum samples in this dissertation and is currently being used in ongoing studies evaluating LEV and PHY pharmacokinetics in pediatric patients following CA.

2.2 MATERIALS AND METHODS

2.2.1 Materials

HPLC-grade methanol was purchased from Fisher Scientific (Pittsburgh, PA). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO). Other aqueous solutions were prepared using ultrapure (double-distilled) water. PHY and LEV were purchased from Sigma-Aldrich (St. Louis, MO). Phenytoin-d_{10} (PHY- d_{10}) and levetiracetam-d_6 (LEV- d_6) were purchased from Cerilliant Corporation (Round Rock, Texas). Drug free serum was purchased from Golden West Biologicals (Temecula, CA).

2.2.2 Patient Population and Serum Sample Collection

The developed assay was applied to serum samples from pediatric patients following CA who were managed using TTM at the Children's Hospital of Pittsburgh. The Institutional Review Board of the Children's Hospital of Pittsburgh approved the protocol. Informed consent was obtained from parents before enrolling the child in the study. At Children's Hospital of Pittsburgh, LEV and PHY are the two first-line therapies administered to children following CA for prophylaxis and treatment of seizures. Since the use of both LEV and PHY are non-protocolized, the choice of therapy and/or dose was at the discretion of the treating physician. In some cases, both LEV and PHY were administered. Blood samples were drawn two times per day on the first four days following cardiac arrest and once on day 7. After centrifugation, serum was stored at -80°C until analysis. Serum levels of LEV and PHY were measured in 20 μL samples according to the assay described in this chapter.

2.2.3 Preparation of Calibration Standards and Quality Control Samples

Two separate stock solutions were prepared for LEV and PHY in methanol at a concentration of 1 mg/mL. These concentrations were spiked into serum to produce stock concentrations containing both LEV and PHY at 0.5, 1, 5, 10, 25, 50 and 75 μg/mL. Standard working solutions were stored in -80°C freezer until use. LEV-d_6 and PHY-d_{10} were used as internal standard (IS). A stock solution containing both internal standards with a final concentration of 1 mg/mL was prepared and stored in -80°C until use. A working solution of IS was prepared fresh from stock solution on day of assay.

For the internal quality control (QC) samples, a separate stock solution was prepared in methanol at a concentration of 2.5 mg/mL. QCs were prepared in serum at three concentrations: 3 μg/mL (low level), 30 μg/mL (medium level), and 60 μg/mL (high level) and stored at -80°C until further use.

To 20 μL of serum, 20 μL of IS working solution (50 ng/mL) was added to an eppendorf tube. For protein precipitation, 200 μL of methanol was added. The sample was vortexed rigorously for 30 seconds and then centrifuged at 13,500 rpm for 8 minutes. Following centrifugation, 20 μL of supernatant was removed and transferred to a new eppendorf tube and then diluted with 200 μL of 50:50 methanol:water. Sample was vortexed for 30 seconds and then 100 μL was transferred to a glass autosampler vial of which 7.5 μL was injected into the UPLC-MS/MS.

2.2.4 Chromatographic and Mass Spectrometric Conditions

The UPLC-MS/MS system consisted of a Waters Acquity Ultra Performance LC coupled to a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Separation of analytical compounds was achieved on a UPLC BEH C18, 1.7 μm (2.1 × 100mm) reversed-phase column (Waters, Milford, MA). The mobile phase consisted of a gradient of solution A (95% water, 5% methanol with 0.05% formic acid) and solution B (10% water, 90% methanol with 0.05% formic acid) with an initial composition of 100% A. Mobile phase composition changed from 100% A to 20% A/80% B at 3 minutes and returned to 100% A at 5 minutes. Total run time was 5 minutes. The flow rate was 0.3 mL/min with a column temperature of 50°C and an injection volume of 7.5 μL.

Analytes were detected via MS/MS with an electrospray ionization source under positive mode with a collision gas pressure of 1.3 mTorr. Mass transitions of LEV (m/z 171→ 125.9), LEV-d_6 (m/z 177→132), PHY (m/z 253.2→182) and PHY-d_{10} (m/z 263.2→192) were optimized. LEV conditions included a spray voltage of 4000 V; collision energy of 13 V; and vaporizer and capillary temperatures of 311°C and 300°C, respectively. PHY conditions included a spray voltage of 3000 V; collision energy of 18 V; and vaporizer and capillary temperatures were both 300°C. Data were acquired and analyzed using XCalibur software version 2.0.6.

2.2.5 Validation Procedures

Validation of the assay in human serum was performed. Seven serum calibration standards ranging in concentration from 0.5 – 75 μg/mL were prepared and analyzed in duplicate in 3 analytical runs on 3 consecutive days. The lower limit of quantification (LLOQ) was determined

by the minimum value with an accuracy and precision within ±15% of the nominal value. The lower limit of detection (LLOD) was determined by the minimum value with a signal-to-noise ratio greater than 3:1.

Accuracy and precision were determined by analyzing QC samples. LEV and PHY were spiked into serum to yield low, medium, and high QCs that corresponded to 3 μg/mL, 30 μg/mL, and 60 μg/mL, respectively. Six samples of each QC were analyzed on days one, two and three of validation and 12 samples of each QC were analyzed on day four of validation. Accuracy was defined as the deviation of the calculated value (E) from that of its true value (T) and expressed as a percentage. This accuracy, or relative standard error (RE%), was calculated using the equation RE% = (E-T)/T*100. This was performed by calculating QC samples on a freshly prepared standard curve. Precision was calculated as the percent coefficient of variance (%CV) where %CV = standard deviation/mean x 100. A deviation and precision within ±15% of the nominal value is considered acceptable.

Stability of the analytes was tested for 6 hours at room temperature and compared with freshly spiked serum. Long-term stability was evaluated using low, medium, and high QCs that had been stored at -20°C and -80°C for 1 month and 3 months.

2.2.6 Evaluation of Matrix Effects and Recovery Efficiency

Matrix effects were evaluated using 0.5 mL volume of the same serum that was used to process calibration curves. Matrices were protein precipitated using 200 μL methanol via the same method described above. Neat samples containing the same concentration of PHY, LEV, PHY-d_{10} and LEV-d_6 were prepared in 50:50 methanol:deionized water. Samples were analyzed using

UPLC-MS/MS as described above. The internal standard normalized matrix factor (IS-normalized MF) for each sample was calculated based on the area ratio (analyte/internal standard) of the post-extraction spiked samples to the neat samples as described by Matuszeki *et al.* IS-normalized MF results are expressed as average percentage ± coefficient of variation (CV) (*n*=5). Values less than 100% indicate ion suppression and values greater than 100% indicate ion enhancement. Recovery efficiency was calculated as the average peak areas spiked after precipitation to before precipitation and expressed as percentage.

2.2.7 Comparison of Published and Modified Quantitation Methods

To date, several methods have been developed to quantify LEV (Pucci, Bugamelli *et al.* 2004, Rao, Ravi *et al.* 2004, Martens-Lobenhoffer and Bode-Boger 2005) or PHY (Haroon and Keith 1983, Kouno, Ishikura *et al.* 1993, Cwik, Liang *et al.* 1997) via HPLC. However, these methods require larger sample volumes and longer run times.

More recently, several studies have reported the quantification of single or multiple AEDs via UPLC-MS/MS (Blonk, van der Nagel *et al.* 2010, Shibata, Hashi *et al.* 2012, Karinen, Vindenes *et al.* 2015). Blonk *et al.* developed a sensitive and fast UPLC-MS/MS method to quantify only LEV in pediatric sample volumes of 50 μL. While this method is highly sensitive and specific for LEV, it is only able to quantify a single drug and would not be applicable to pediatric patients who receive combination therapy. Shibata *et al.* developed a method to quantify LEV and PHY, among many other AEDs, in 50 μL plasma samples using UPLC-MS/MS. To the best of our knowledge, this is the smallest sample volume which quantifies AEDs via UPLC-MS/MS. However, the total run time for this assay was 10 minutes per sample

and no internal standards were used in this development or processing with this assay. Therefore, we aimed to expand on these current methods to develop an assay with greater sensitivity (20 μL per sample) and a shorter run time (< 5 mins per sample).

2.2.8 Statistical Analysis

Statistical analysis was completed using GraphPad Prism software, version 4.03 (GraphPad Software, La Jolla, CA). In the matrix effects studies, IS-normalized MF values were compared using unpaired t-test (2 tailed). For all statistical tests, a $p<0.05$ was considered significant.

2.3 RESULTS

The following section reports the results of the development, linearity, accuracy, precision and validation of the UPLC-MS/MS assay.

2.3.1 Development of UPLC-MS/MS Method

Mass spectrometer parameters were optimized to achieve complete fragmentation (**Table 4**) and chromatographic conditions were optimized to achieve short run time and optimal peak shape. The optimal mobile phase consisted of solution A (95% water, 5% methanol with 0.05% formic acid) and solution B (10% water, 90% methanol with 0.05% formic

acid). A representative chromatogram of LEV (25 μg/ml), PHY (25 μg/ml), LEV-d_6 (50 ng/mL) and PHY-d_{10} (50 ng/mL) is depicted in

Figure 2. The elution sequence was as follows: LEV (2.35 mins), LEV-d_6 (2.34 mins), PHY (3.80 mins), and PHY-d_{10} (3.78 mins).

Table 4: UPLC-MS/MS conditions for the quantification of LEV and PHY.

Parameter	LEV Setting	PHY Setting
Run duration (min)	4	4
Vaporizer temperature (°C)	311	300
Capillary temperature (°C)	300	300
Spray voltage (V)	4000	3000

	LEV	LEV-$_{d6}$	PHY	PHY-$_{d10}$
Q1 (*m/z*)	171.0	177.0	253.2	263.2
Q3 (*m/z*)	125.9	132.0	182.0	192.0
Collision energy (V)	13	13	18	18

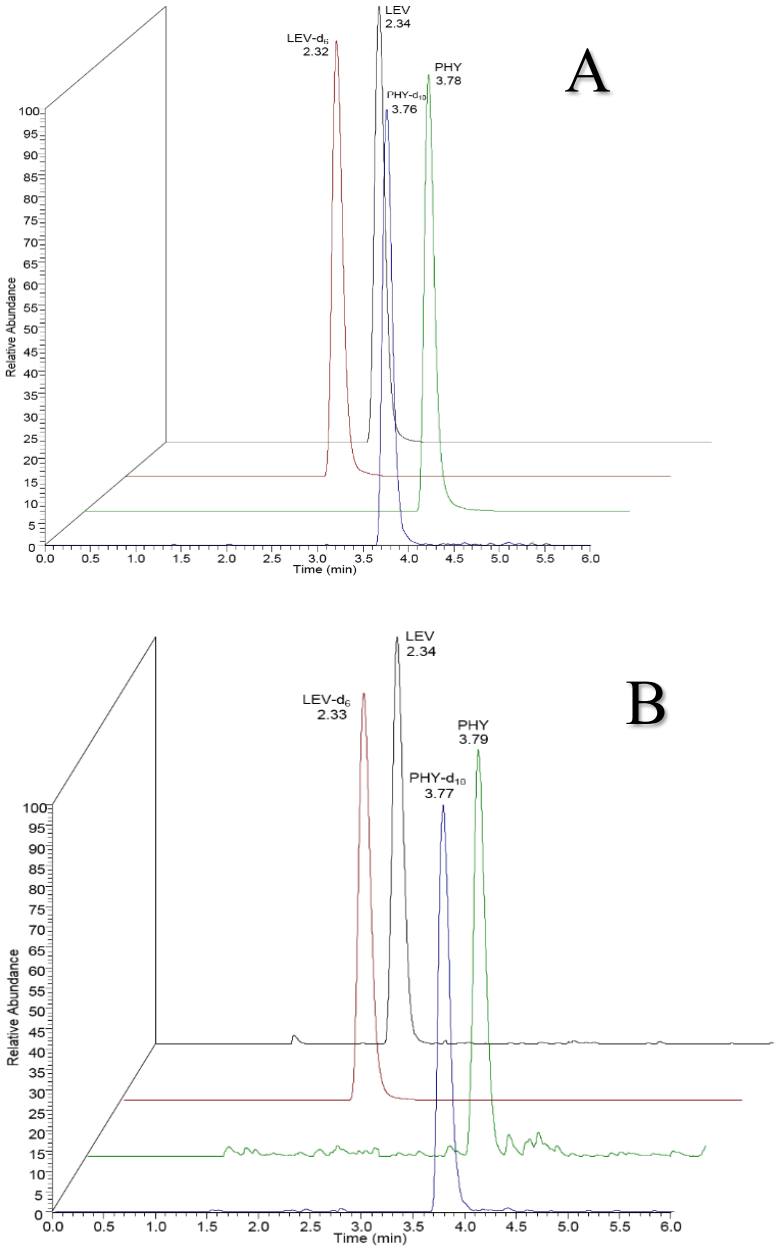


Figure 2: Chromatograms of drug analytes and internal standards.

(A) spiked human serum at the 50 µg/ml standard. Levetiracetam (50 µg/ml), levetiracetam-d_6 (50 ng/ml), phenytoin (50 µg/ml), and phenytoin-d_{10} (50 ng/ml) and (B) spiked human serum at the LOQ. Levetiracetam (5 µg/ml), levetiracetam-d_6 (50 ng/ml), phenytoin (5 µg/ml), and phenytoin-d_{10} (50 ng/ml).

2.3.2 Linearity, Accuracy, and Precision

We performed a validation of the calibration curves which provide a reliable and linear range for both LEV (0.5 to 75 µg/mL) and PHY (0.5 to 75 µg/mL). **Figure 3** shows the calibration curves for both drugs. The correlation coefficients of the 1/y-weighted calibration curves were in the range of 0.9975 – 0.9991 and 0.9975 – 0.9991 for PHY and LEV, respectively. The lower limit of quantification in this assay was 0.5 µg/ml for levetiracetam and phenytoin. Even with the very low serum volumes, we were still able to achieve adequate sensitivity with an estimated LOD of 0.02 µg/mL for both PHY and LEV, which is well below the LLOQ at 0.5 µg/mL.

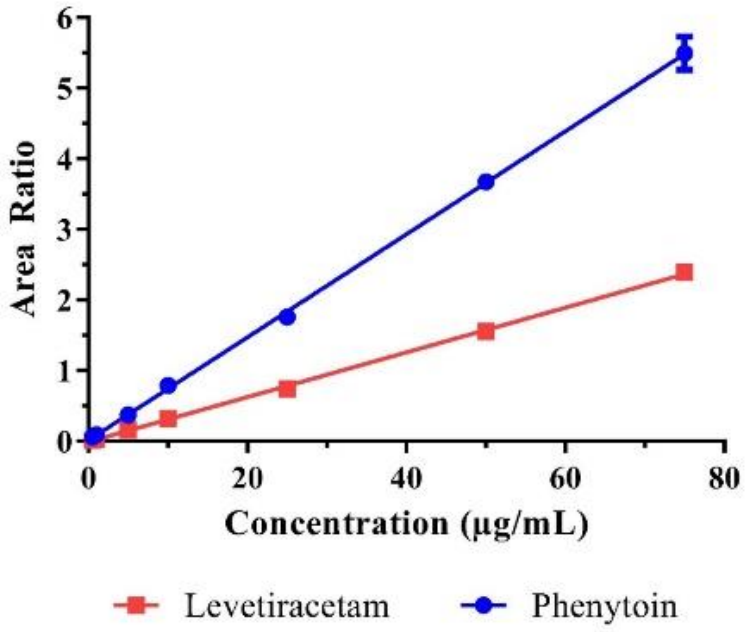


Figure 3: Validated calibration curves for PHY and LEV.

The intra-day and inter-day precision and accuracy of the assay was less than 11.9% at each QC level. Results of the assay validation are presented in **Table 5**. Intra- and inter- assay precision and accuracy were within the acceptable range of 15% for both LEV and PHY.

Table 5: Accuracy and precision of assay.

	Levetiracetam			Phenytoin		
Nominal concentration (μg/mL)	Mean calculated conc (μg/mL)	Accuracy (% deviation)	Precision (%CV)	Mean calculated conc (μg/mL)	Accuracy (% deviation)	Precision (%CV)
Intra-assay[a]						
3	3.04	1.25	10.42	3.27	9.16	7.95
30	31.51	5.03	7.59	33.48	11.60	2.01
60	56.23	-6.28	3.76	59.82	-0.30	7.83
Inter-assay[b]						
3	2.83	-5.71	10.92	3.03	0.88	11.90
30	29.04	-3.20	10.54	31.02	3.39	10.20
60	61.72	2.87	9.53	63.48	5.80	7.99

[a] n = 12 (one run)

[b] n = 24 (three different runs of n=6 performed on separate days)

The bench-top and long-term stability studies demonstrated that the analytes were stable for up to 6 hours at room temperature and for 1 month and 3 months at -20°C and -80°C. The average percent change of peak areas were less than 4.8%.

2.3.3 Assay Validation

This assay was successfully applied to 30 serum samples from twelve pediatric patients following cardiac arrest who were being managed using therapeutic hypothermia and receiving LEV and/or PHY. The dose administered ranged from 5 - 38 mg/kg for levetiracetam (n = 6 patients) and 2.5 – 20 mg/kg for phenytoin (n = 6 patients). PHY and LEV concentrations from these varying doses ranged from 11.38 – 27.09 μg/ml and 0.66 – 89.53 μg/ml, respectively. All serum samples fell above our LLOQ thus confirming the suitability of the assay range for this patient population (**Figure 4**).

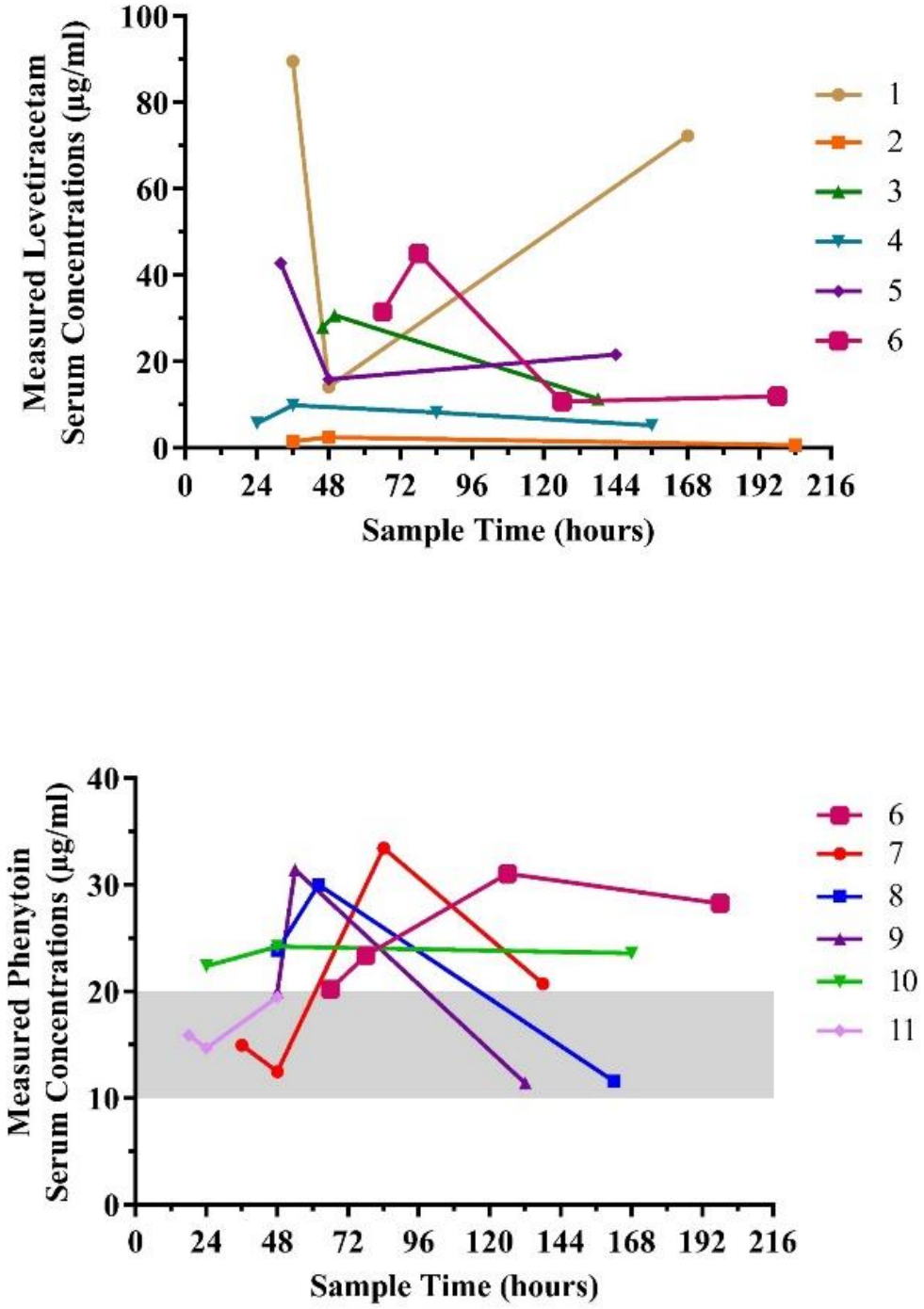


Figure 4: Quantitation of PHY and LEV in serum of pediatric patients following CA.

2.3.4 Analysis of Matrix Effects

The matrix effect in serum was between 106.0 - 109.4% and 107.4 – 109.2% for LEV and PHY, respectively at all 3 QC levels. This demonstrates minimal matrix effects of serum on the reproducibility and reliability of the assay. The recovery in serum ranged from 98.6 – 99.8% and 98.6 – 99.2% for LEV and PHY, respectively. **Table 6** shows the results of the matrix effect and recovery efficiency.

Table 6: Evaluation of matrix effects and recovery efficiency.

	LEV			LEV-d_6	PHY			PHY-d_{10}
	3 µg/mL	30 µg/mL	60 µg/mL	50 ng/mL	3 µg/mL	30 µg/mL	60 µg/mL	50 ng/mL
ME % (%CV)	106.0 ± 2.8	108.1 ± 1.8	109.4 ± 2.2	101.2 ± 2.1	108.9 ± 3.3	109.2 ± 3.5	107.4 ± 2.4	102.1 ± 1.8
RE % (%CV)	99.8 ± 2.6	99.4 ± 2.2	98.6 ± 2.7	106.2 ± 3.2	98.6 ± 2.5	99.2 ± 1.7	98.7 ± 2.7	104.2 ± 2.9

2.4 CONCLUSIONS

A protein precipitation sample preparation combined with a UPLC-MS/MS method was validated and successfully applied for simultaneous determination of PHY and LEV. This

method was optimized from previous reports to achieve high sensitivity for the detection of these two drugs in small serum volumes of 20 μL. A linear calibration curve was validated from 0.5-75μg/ml. This method was successfully used to measure PHY and LEV in pediatric serum samples that fell above the LLOQ. This method is the first to report a quick and easy method to quantify both PHY and LEV in small serum volumes as low as 20 μL. Further, this assay method was successful applied on samples obtained from patients in a realistic clinical setting where one or both of the drugs were being administered and where therapeutic hypothermia and critical care therapies were being applied.

2.5 DISCUSSION

In this study we demonstrated that this method is useful in simultaneously measuring phenytoin and levetiracetam in low volumes of serum samples. Both AEDs, and their respective IS, were simultaneously detected, identified, and quantified using a validated UPLC-MS/MS method. Furthermore, we determined that these two drugs are generally stable for 24 hours at room temperature. Collectively, these results suggest that UPLC-MS/MS is an accurate and sensitive method for quantification of phenytoin and levetiracetam in serum samples of 20 μL.

More specifically, this chapter describes the validation of linear calibration curves ranging from 0.5 – 75 μg/mL. The inter-day and intra-day variance was less than 15% at all concentrations with a recovery efficiency of greater than 98%. Additionally, we demonstrated that the matrix effect of serum did not significantly affect the reliability and reproducibility of the assay, for both compounds measured.

Our results also demonstrate that phenytoin and levetiracetam are stable at room temperature in serum for up to 6 hours. Further, both phenytoin and levetiracetam were stable under -20°C and -80°C for 1 month and up to 3 months. Collectively, these results suggest that serum samples stored in -80°C freezer and then analyzed at room temperature for less than 6 hours are appropriate for quantification of phenytoin and levetiracetam.

3.0 POPULATION PHARMACOKINETICS OF PHENYTOIN IN PEDIATRICS FOLLOWING CARDIAC ARREST

3.1 INTRODUCTION

As discussed in Chapter 2, pediatric patients are at high risk to develop seizures as a result of brain insult following CA. For this reason, pediatrics typically receive AEDs prophylactically to prevent the onset of CA-induced seizures. Multiple factors can affect drug metabolism in critically ill patients including ischemia during cardiac arrest, targeted temperature management, inflammation, and co-administered medications, to name a few. Previously our laboratory demonstrated an important need to monitor AEDs in pediatrics following TBI, particularly during the hypothermic and re-warming phases of therapeutic hypothermia. The results of this population pharmacokinetic (PopPK) analysis are discussed in the subsequent section.

3.2 BACKGROUND ON POPULATION PHARMACOKINETIC ANALYSIS IN CHILDREN WITH TBI

In this prospective clinical trial, children with severe TBI (less than 18 years of age) were randomized to receive 48 hours of therapeutic hypothermia (n = 10) or normothermia standard-of-care treatment (n = 9) (Empey, Velez de Mendizabal *et al.* 2013). Pediatric patients were enrolled as part of a clinical trial to determine the effect of induced moderate hypothermia (32-33 °C) on outcomes in children after severe TBI ("Cool Kids Trial," Clinicaltrials.gov: NCT00222742). Phenytoin is a commonly administered AED in children undergoing therapeutic hypothermia, however little is known about its pharmacokinetics during cooling. Thus, a population pharmacokinetic analysis was performed to describe phenytoin disposition during cooling using nonlinear mixed-effects modeling (NONMEM).

The pharmacokinetics of phenytoin in this study was best described using a 1-compartment Michaelis-Menten model with parameter estimates for the Michaelis-Menten elimination rate constant (K_m) and maximum velocity of metabolism (V_{max}). Results of the population pharmacokinetic modeling showed that therapeutic hypothermia decreased the V_{max} of phenytoin elimination by as much as 49.5%. An important finding of this study was that hypothermia-mediated effects on phenytoin PK continued well past the cooling period and into the rewarming and post-treatment phases of treatment. Collectively, these results demonstrated that therapeutic hypothermia led to an increase in phenytoin levels during the cooling and rewarming phases due to an overall decrease in phenytoin elimination in children with TBI (Empey, Velez de Mendizabal *et al.* 2013). This study is the first to use a robust PopPK approach to quantify the effect of hypothermia on phenytoin metabolism. We aimed to expand on these results in children with severe TBI to another patient population, pediatrics with CA, of which therapeutic hypothermia is recommended and commonly used as a neuroprotective therapy (Bernard, Gray *et al.* 2002, Shankaran, Laptook *et al.* 2005).

3.3 AIMS AND HYPOTHESIS OF STUDY

We aimed to investigate the effects of therapeutic hypothermia on the pharmacokinetics of phenytoin in pediatrics following a cardiac arrest using a PopPK analysis. We hypothesized that therapeutic hypothermia would lead to an increase in phenytoin levels due to a decrease in overall phenytoin elimination, as was seen in children with TBI. However, as discussed in Chapter 1, the extent of injury-mediated versus hypothermia-mediated effects on PK is still largely unknown, and thus the degree of hypothermia effects on phenytoin elimination may be

vastly different across patient populations. The following sections describe the clinical study methods and the population pharmacokinetic analysis performed to investigate therapeutic hypothermia in pediatrics with CA.

3.4 SUBJECTS AND METHODS

Patients were recruited and enrolled under an IRB approved protocol as part of multiple clinical studies investigating therapeutic hypothermia in pediatrics with CA. Pediatric patients were pooled from a study which aimed to investigate the optimal duration of hypothermia for brain protection in children following cardiac arrest (K23 IRB #PRO07080349, Title: What is the optimal duration of hypothermia for brain protection in children following cardiac arrest?). In this study, patients (ages 2 days - 18 years) suffering a CA requiring chest compressions for at least 2 minutes with ROSC were randomized to receive normothermia or hypothermia (33°C) treatment for 24 or 72 hours.

Additionally, patients were pooled from a clinical trial which aimed to evaluate the effect of therapeutic hypothermia on clinical outcomes in pediatrics with CA. Furthermore, a number of patients were enrolled through a separate study which aimed to investigate biomarkers in pediatric CA patients. All serum samples were obtained from one of these three clinical studies and under IRB approval. Following serum collection, an IRB exemption was granted to achieve additional demographic and covariate information for these subjects.

3.4.1 Drug administration

Anti-epileptic drugs are often used as prophylactic therapy in children following CA to prevent the onset of seizures. At our institution, the two most commonly administered AEDs in pediatrics with CA are phenytoin and levetiracetam, of which the selection of AED is non-protocolized. In some cases, pediatrics received either phenytoin or leveitracetam, while in other cases pediatrics received a combination of one followed by the other, depending on seizure activity and response to treatment. In other cases, pediatrics were administered one of the AEDs not as prophylaxis but in response to seizure activity. Fosphenytoin is administered as an intravenous loading dose of 10-20 mg/kg followed by maintenance therapy. Clinical data collection included amount and time of fosphenytoin and levetiracetam doses, and any concurrent medications.

3.4.2 Serum Sampling, Therapeutic Drug Monitoring, and Data Collection

Serum sampling times for PK analysis were designed prospectively. Serum PK samples were taken twice a day on Days 1-4 and on Day 7 following cardiac arrest. Sampling times on Days 1-4 were designed to capture the cooling and rewarming phases of hypothermia treatment while Day 7 was chosen to capture the post-treatment phase. The exact date and time when each serum sample was drawn was recorded.

In addition to serum PK samples, blood samples were collected for routine therapeutic drug monitoring (TDM) as part of clinical care. Concentrations were measured at the Children's Hospital of University of Pittsburgh Medical Center using a particle-enhanced turbidimetric inhibition immunoassay (Beckman-Coulter, Brea, CA) with or without initial sample ultrafiltration. Both total and free phenytoin levels were measured as part of TDM, and used to

guide dosing of fosphenytoin to maintain therapeutic ranges. The date and time of each TDM measurement was recorded. Further, all study times were reported from time of injury.

Demographic and covariate information was also collected via chart review. Demographic information included age, sex, height, and weight. Covariate information included extensive temperature data (hourly or more frequent); co-administered medications (fentanyl and levetiracetam); indices of organ function including albumin (ALBL), hepatic albumin (ALBH), blood urea nitrogen (BUNH), corticotropin-releasing hormone (CRH), bilirubin (BILIH), alanine aminotransferase (ALTH), and aspartate aminotransferase (ASTH); extra-corporeal membrane oxygenation (ECMO) treatment; and length of cardiac arrest was used as a measure of disease severity.

3.4.3 Hypothermia Protocol

Children were cooled for a duration of 24, 48 or 72 hours. In some cases, children came into the ICU already cooled and in some cases children died before finishing hypothermia treatment. Temperature was monitored using a rectal temperature probe to measure core body temperature. A cooling blanket and/or cold intravenous saline were used to achieve and maintain core body temperature. Following the cooling duration, patients were slowly rewarmed over a 24 – 48 hour period until normal body temperature was achieved.

Since cooling duration varied among patients and to maintain consistency across studies, the following treatment periods were designated based on length of cooling and initial time of administration. For children who underwent 24 hours of cooling, the cooling phase was designated between 10-34 hours; the rewarming phase was designated between 34-72 hours; and the post-treatment phase was designated after 72 hours. For children who underwent 72 hours of

cooling, the cooling phase was designated as 12-84 hours; the re-warming phase was designated as 84-104 hours; and the post-treatment phase was designated after 104 hours.

Figure 5 and **Figure 6** show the compiled temperatures across the children who were cooled for 24 or 72 hours, respectively. These treatment periods were used for initial exploratory data analysis. The differences in hypothermia treatment across patients is another reason why using a robust PopPK approach is necessary since this allows for temperature to be included as both a continuous and categorical covariate. Individual temperature profiles for all patients are included in **Appendix A.1**.

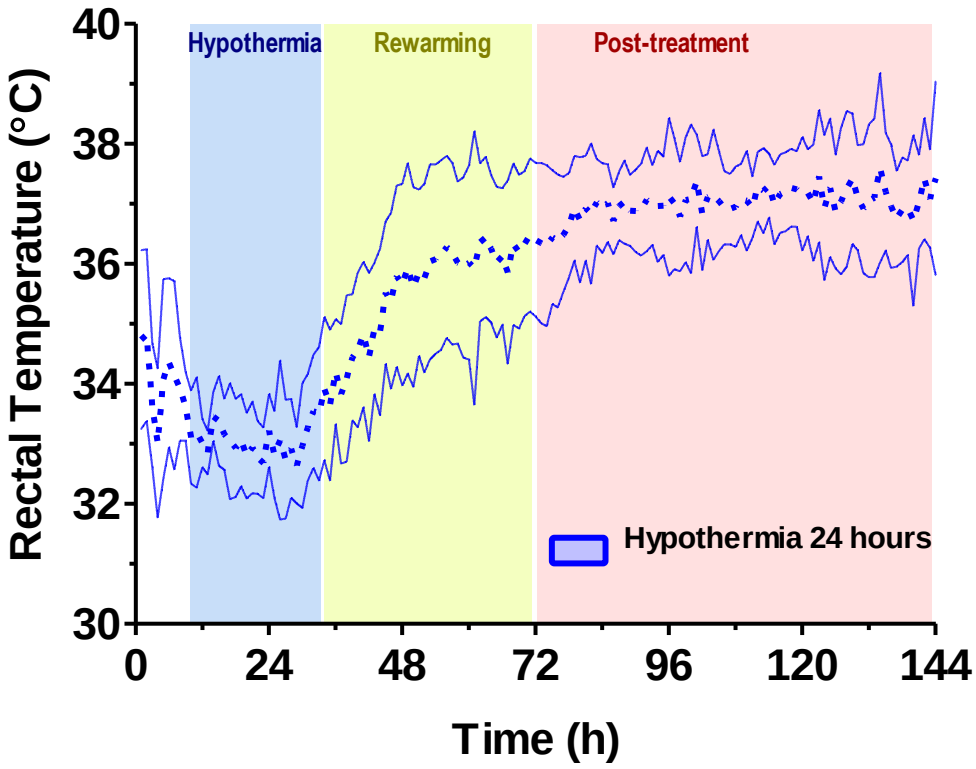


Figure 5: Temperature profiles of patients who were cooled for 24 hours.

Protocol timing is organized into three designated shaded areas: hypothermia; rewarming; and post-treatment. Rectal temperature designates the success of the study protocol in achieving 24 hours of hypothermia treatment. Straight lines represent the 95% confidence intervals.

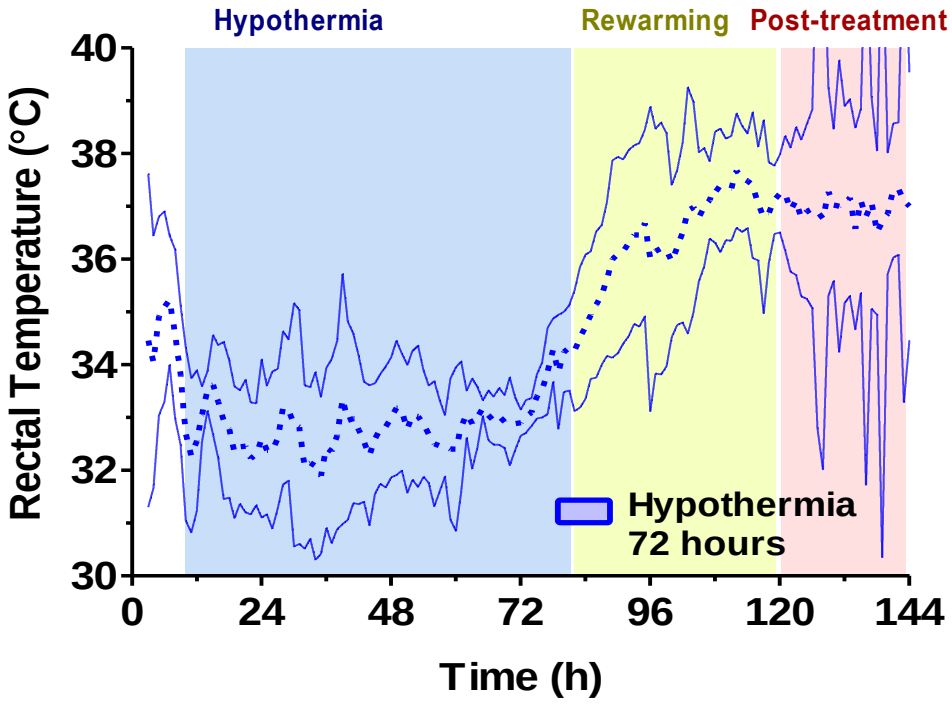


Figure 6: Temperature profiles of children who were cooled for 72 hours.

Protocol timing is organized into three designated shaded areas: hypothermia; rewarming; and post-treatment. Rectal temperature designates the success of the study protocol in achieving 72 hours of hypothermia treatment. Straight lines represent the 95% confidence intervals.

3.5 DATA COMPILATION

A NONMEM input file (**Appendix B.1.**) for the population PK analysis was constructed to contain the full dosing records and PK observation records. Phenytoin levels measured as part of routine TDM in the clinic and from levels quantified via UPLC-MS/MS were both included. Relevant covariates for each individual were also included. An explanation of each variable in the NONMEM input data file and its description is described in **Appendix B.2.**

3.6 DATA REVIEW

Serum phenytoin level measurements excluded from the analysis were retained in the dataset and commented out (C = C). Missing phenytoin levels or doses were not imputed or accounted for in the analysis. All serum phenytoin levels which were below the limit of quantification (BLQ) were excluded from the analysis. Missing covariate information was denoted as missing in the dataset.

3.7 POPULATION PHARMACOKINETIC METHODS

NONMEM® program Version 7.3 was used in this analysis (ICON Development Solutions, Ellicott City, Maryland, USA) [**Error! Reference source not found.** Users Guides, 2009]. irana (version 2.9.4) was used as the NONMEM interface. PK parameters were estimated using the First-Order Conditional Estimation Method with Interaction (FOCEI) method. Perl-speaks-

NONMEM (PsN, version 4.6.0) was used for NONMEM execution and VPC. R (version 3.2.3; http://r-project.org), R Studio (version 0.98.1091; https://www.rstudio.com/) and Prism (version 5.0) were used for post-processing and plotting of results.

3.7.1 Population Pharmacokinetic Analysis

Prior knowledge of the compartmental disposition of serum phenytoin derived from the previous PopPK analysis in children with TBI (**3.2**) suggested that a 1-compartmental Michaelis-Menten model with a time-variant maximum velocity term provides an adequate description of phenytoin concentration-time data. Therefore, this model served as a starting point for the present analysis, and was tested during development of the structural model. The disposition parameters were expressed in terms of volume of distribution (V_1), time varying Michaelis-Menten parameter at baseline (V_{max0}), Michaelis-Menten elimination rate constant (K_m), maximum velocity of metabolism (V_{max}), and proportionality constant between bound and unbound drug (θ_{prop}).

In addition to the 1-compartment Michaelis-Menten model, a linear 1-compartment and 2-compartment model were also tested as previous population pharmacokinetic analysis have used these models to describe phenytoin PK (Tanaka, Kasai *et al.* 2013, Hennig, Norris *et al.* 2015). Previous modeling has indicated that it may be advantageous to use log-transformed data; hence both log-transformed and untransformed phenytoin serum levels were explored.

3.7.2 Pharmacostatistical Model

Distributions of individual parameters (P_i) were assumed to be log-normal and were described by an exponential error model:

$$P_i = \hat{P}\exp(\eta^{Pi})$$

where: P_i is the parameter value for individual *i,* $\hat{P}$ is the typical population value of the parameter (when eta=0), and η^{Pi} are individual-specific inter-individual random effects for individual *i* and parameter *P* that are assumed to be normally distributed ($\eta \sim N(0, \omega^2)$) with covariance's defined by the inter-individual covariance matrix Ω.

Initial model building was performed using diagonal covariance matrix of inter-individual random effects. For the final model, attempts were made to define a full block covariance matrix for the inter-individual random effects (Ω). If the resulting model was not numerically stable and the goodness of fit criteria demonstrated no clear difference, then the preference was given to models with on-diagonal elements.

For PK observations in this analysis, the residual error model was a combined additive and proportional error model:

$$C_{ij} = \hat{C}_{ij}(1+\varepsilon_{pij}) + \varepsilon_{aij}$$

where C_{ij} is the *j*th measured observation in individual *i,* $\hat{C}_{ij}$ is the *j*th model predicted value in individual *i,* and ε_{pij} and ε_{aij} are proportional and additive residual random errors, respectively, for individual *i* and measurement *j*. They were assumed to be independently and identically distributed: $\varepsilon_x \sim NID\ (0, \sigma_x^2)$.

3.7.3 Covariate Model

After the development and selection of the best structural model, covariate modeling was performed for phenytoin levels on V_{max} and K_m only. The final model was then used to model phenytoin levels.

The following continuous covariates were considered: body weight (WT), age, length of cardiac arrest, and temperature.

The following categorical covariates were considered: age (< 1 year versus ≥ 1 year), gender, co-administered drugs (fentanyl and levetiracetam), temperature (hypothermic versus normothermic).

The full model with backward deletion approach was utilized for covariate modeling. An initial exploratory assessment of covariate effects versus predicted etas from the base model was performed as part of covariate screening. A full model was then developed with all covariates of interest. Backward deletion was carried out at the $p < 0.001$ (increased objective function value (OFV) less than 10.83 points, d.f. = 1) significance level where the relative influence of each covariate on the model was re-evaluated by deleting it from the semi-full model on an individual basis. Backward deletion was carried out until all remaining covariates in the model were significant at $p < 0.001$.

Continuous covariates (COV) were centered at their typical values (TV_{COV}) and typical population value (TV_P) expressed as:

$$TV_P = \theta_P \cdot \left(\frac{COV_i}{TV_{COV}} \right)^{\theta_{COV,P}}$$

where TV_P and TV_{COV} are as defined above, θ_P is the estimated parameter representing the typical value of model parameter P when the individual covariate (COV_i) is equal to TV_{COV} and $\theta_{COV,P}$ is the estimated parameter representing the influence of covariate COV on model parameter P.

Categorical covariates (CAT) were tested and incorporated in the model as a series of index variables taking on values of zero or one (*e.g.*, CAT_1, CAT_2, …, CAT_{n-1} representing the n-1 levels of CAT). Index variables were included in the model as follows:

$$TV_p = \theta_p \cdot \prod_{i=1}^{n-1} \left(\theta_{CAT_{i,P}} \right)^{CAT_i}$$

where TV_P is as previously defined, θ_P is the estimated parameter representing the typical value of model parameter P for a reference category when all the individual categorical covariate index variables (CAT_i) are equal to zero and $\theta_{CAT_{i,P}}$ is the estimated parameter representing the relative influence of a categorical covariate index variable on model parameter P when CAT_i is equal to one.

3.8 MODEL EVALUATION AND DISCRIMINATION

3.8.1 Goodness of Fit

The goodness-of-fit (GoF) for a model was assessed by a variety of plots and computed metrics:

- Observed versus population and individual predicted concentration plots;
- Conditional weighted residuals (CWRES) versus population predicted concentrations and versus time plots;
- Histograms of individual random effects to ensure they were centered at zero without obvious bias;
- Scatter plots of individual random effects versus modeled covariates;
- Relative standard errors (RSE) of the parameter estimates;
- Shrinkage estimates for each η and ε;
- Successful minimization and execution of a covariance step;
- The minimum objective function value (OFV).

The difference in the objective function value (ΔOFV) between models was considered proportional to minus twice the log-likelihood of the model fit to the data and was used to compare competing hierarchical models. Models were considered hierarchical if the more complex model could be reduced to the less complex model by removal of one or more model parameters. This ΔOFV was asymptomatically χ^2 distributed with degrees of freedom (d.f.) equal to the difference in number of estimated parameters between the two models. A ΔOFV with a χ^2 probability less than or equal to 0.01 (6.64 points of OFV, d.f. = 1) would favor the model with the lower OFV. Backward elimination during covariate evaluation used a more stringent criterion at a significance level of less than or equal to 0.001 (10.83 points of OFV, d.f. =1).

3.8.2 Visual Predictive Checks

The final phenytoin population PK model was also evaluated by performing a prediction corrected (pc) VPC (**Error! Reference source not found.** *et al.*, 2011) to assess how closely odel simulations replicated both the central tendency and the variability in the observed data. As such, the predicted median, 5^{th}, and 95^{th} percentiles of the concentration time courses following 1000 simulations were superimposed with the observed data.

3.8.3 Simulations

Using results of the final PopPK model of phenytoin, simulations were performed to determine phenytoin elimination under clinically relevant conditions. A 1000 pediatrics receiving a standard loading dose of fosphenytoin (20mg/kg) followed by maintenance doses and cooled to 33°C for 24 or 72 hours was simulated. A population median and 90% confidence interval was determined.

3.9 RESULTS

3.9.1 Dataset Analyzed

A total of 36 pediatrics with cardiac arrest were included in the analysis dataset. Of the 36 patients, two subjects were undergoing ECMO treatment; three subjects were cooled previous to start of study; and eight subjects died before the end of hypothermia treatment.

3.9.2 Phenytoin Concentrations

Of the phenytoin concentrations measured as part of TDM (total PHY: n = 178; free PHY: n = 162), one level was excluded because it fell well above a normal phenytoin level (55 µg/mL). **Figure 7** depicts the total phenytoin levels from TDM measurements against time after first dose (TAFD). Overall, the phenytoin levels remain fairly consistent following first dose; however it is difficult to tease out treatment phases since dosing began at different phases across all patients. Each patient had an average of 7 serum samples (range: 4 – 10 samples per patient).

Further, of the phenytoin concentrations measured via UPLC-MS/MS, all samples post-dose were quantifiable via UPLC-MS/MS with respect to phenytoin concentrations. However, 8% of pre-infusion samples were BLQ. A total of 29 serum samples were quantified from 4 subjects who received only phenytoin and a total of 77 serum samples were quantified from 10 subjects who received both phenytoin and levetiracetam.

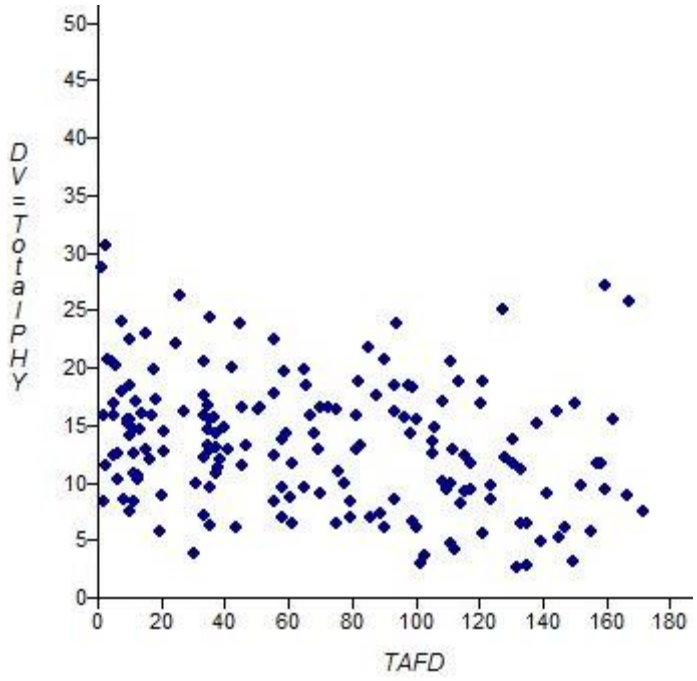


Figure 7: Total phenytoin concentration (µg/mL) versus time after first dose (hours) as measured by routine therapeutic drug monitoring.

3.9.3 Demographics and Covariates

Table 7 presents the basic distribution of covariates. Demographic data were expressed as mean ± standard deviation (SD) unless the data were not normally distributed, in which case median and range were reported.

One subject was a neonate (age = 0.04 years; 14.2 days old); ten subjects were infants (between 0.07 to 1 year old); and the remaining twenty-two subjects were children (between 1-18 year old). Three subjects did not have a recorded age in their medical charts.

Of the 36 patients administered phenytoin, 27 (75.0%) of them also received fentanyl. Additionally of the patients receiving phenytoin, 13 (36.1%) also received levetiracetam. In

some cases, levetiracetam was administered simultaneously with phenytoin and in other cases levetiracetam was administered prior or following treatment with phenytoin.

In this study, length of cardiac arrest, defined as time from collapse to return of spontaneous circulation, was used as a measure of injury severity. Studies have demonstrated that a cardiac arrest longer than 25 minutes is generally associated with poor outcomes (Hayakawa, Tasaki *et al.* 2011). The median length of cardiac arrest in this study was 27 minutes (range: 3 – 70 minutes). Sixteen subjects had a cardiac arrest longer than 25 minutes. Fifteen subjects had a cardiac arrest 25 minutes or less. Five subjects did not have a recorded length of cardiac arrest.

Table 7: Subject characteristics and demographics.

Male – n (%)	12 (33.3%)
Height (cm) - mean (SD)	99.91 (38.41)
Weight (kg) - mean (SD)	23.54 (21.42)
Age (yr) - median (range)	2.44 (0.04)
CPR (mins) – median (range)	27 (3 – 70)
Albumin	
Day 1 - mean (SD)	2.71 (0.74)
Day 2 - mean (SD)	3.11 (0.60)
Day 3 - mean (SD)	2.83 (0.77)
Day 7 - mean (SD)	3.43 (0.54)
AST - median (range)	104 (29 – 4191)
ALT - median (range)	61 (1 – 3624)
CRH – median (range)	0.3 (0.07 – 10)
BUNH – median (range)	13 (2 – 93)
Co-Administered Drugs	
Fentanyl – n (%)	27 (77.1%)
Levetiracetam	13 (37.1%)

Footnote: BUNH: blood urea nitrogen; CRH: corticotropin-releasing hormone; ALT: alanine aminotransferase; AST: aspartate aminotransferase.

3.9.4 Phenytoin Population Pharmacokinetic Model Development

A summary of the phenytoin population PK model development is presented in **Table 8**. A one-compartment Michael-Menten model provided a good description of the phenytoin concentration-time data. The phenytoin doses were administered as a short IV injection and hence dosing events were coded as infusions in the NONMEM dataset.

3.9.4.1 Base Model Results

As listed below in the following table, the final base model was a one-compartment Michaelis-Menten model with parameters for the elimination rate constant (K_m) and maximum velocity (V_{max}). A subset of the base models tested are listed in **Table 8**.

Table 8: Summary of models run when development structural PopPK model of phenytoin.

Run Number (in Pirana)	Description of Model	OFV	Comment
2	1-cmpt model	-43.823	
11	1-cmpt Michaelis Menten Model	-363.853	
12	Added time-variant V_{max} parameter	-391.062	
18	**Added generalized additive model (GAM)**	**-482.937**	**GAM decreased overall OFV**
19	Added omega block	-481.883	
22	2-cmpt model	-98.475	
29	Proportional Error Model	-326.843	
30	Combined Error Model	-371.912	
78	Combined TBI and CA patient populations	-906.091	
81	Combined CA patients with normothermic TBI patients	-595.893	

Final base model highlighted in bold.

The 1-compartment Michaelis-Menten model to describe phenytoin PK was built as follows:

Equation 1: $\frac{dUnb}{dt} = -V_{max} * \frac{Unb/V_1}{k_m + Unb/V_1}$

Equation 2: $Bou = \theta_{prop} * Unb$

Equation 3: $Total_{drug} = Unb + Bou$

Equation 4: $V_{max}(t) = V_{max0} + V * (1 - e^{-k_{ind} * t})$

where Unb is the amount of free phenytoin; Bou is the amount of boud phenytoin; V_{max} is the maximum velocity of metabolism; k_m is the Michaelis-Menten elimination constant; V_1 is the volume of distribution in liters; k_{ind} is the rate constant describing induction; θ_{prop} is the proportionality constant between the bound and unbound drug; V_{max0} is the time invariant maximum velocity of metabolism at baseline; V_{maxt} is the time variant maximum velocity of metabolism; V_{maxi} is the portion of velocity impacted by induction and described by k_{ind}.

Error! Reference source not found. describes the 1-compartment Michaelis-Menten odel that was fit to the unbound phenytoin concentration. Error! Reference source not found. describes the relationship of total and free phenytoin in the model where θ_{prop} is the proportionality between bound and unbound phenytoin. Total phenytoin was the sum of bound and unbound phenytoin (Error! Reference source not found.). Additionally, a time variant

aximum velocity term was incorporated into the final model (Error! Reference source not found.). This equation makes the assumption that V_{max} varies as a function of time.

GoF plots for the base model demonstrate good agreement between the predicted and observed data. There appears to be a deviation in the population prediction activity versus observed activity plot at higher concentrations, which indicates the need to account for covariates.

Correlations between covariates were assessed prior to inclusion in the model and are displayed in **Figure 8**. Due to the inclusion of neonates and infants in the dataset, the relationship between age and weight is also presented in **Figure 9**. As expected, there was a strong correlation between body weight and age in the pediatric population ($R^2 = 0.88$). Based on this, age was tested as a continuous and categorical covariate in the full model (age < 1 years versus age ≥ 1 years).

Based on these exploratory plots as well as on previous phenytoin PopPK modeling and clinical rationale, the following covariates were tested in the full model on the PK parameters: age, weight, height, gender, temperature and length of cardiac arrest. The inclusion of these covariates constituted the "full model" as discussed below.

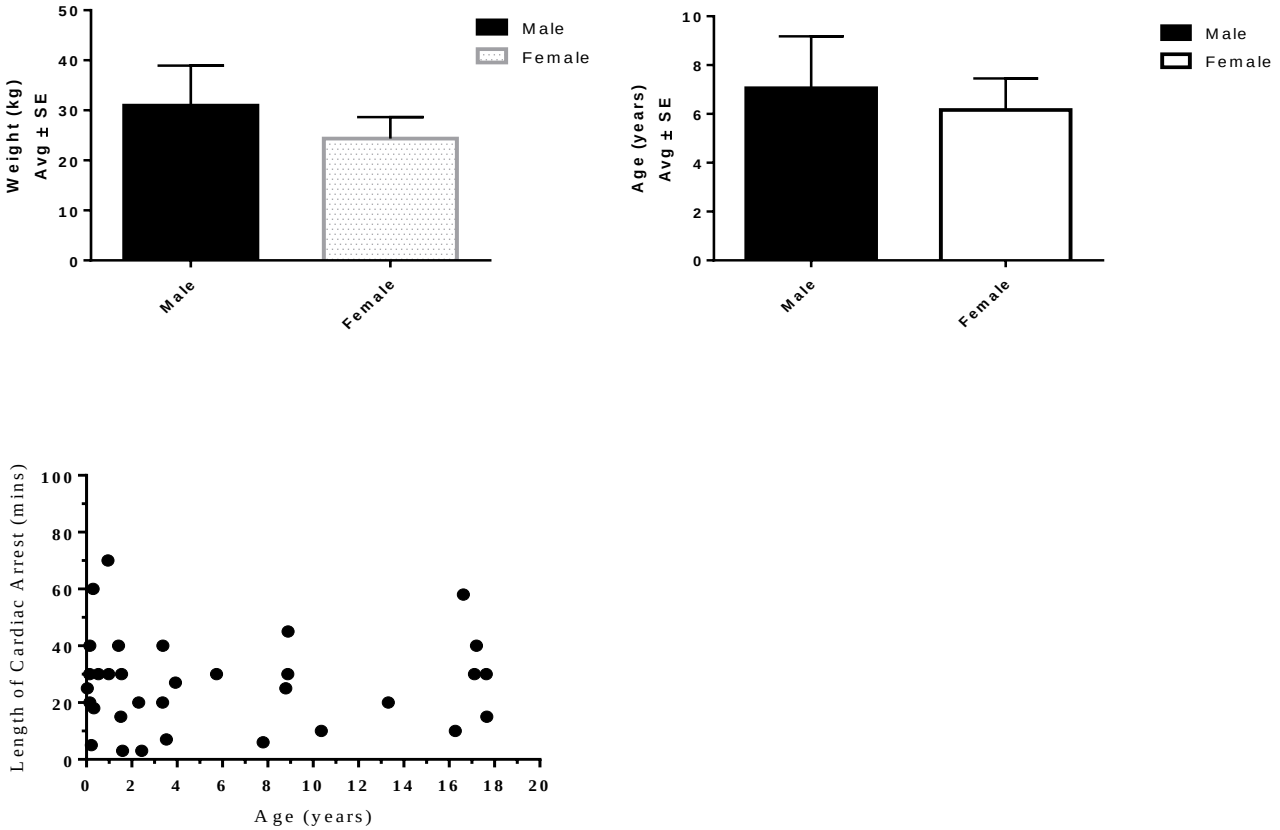


Figure 8: Comparison between weight and age in males versus females (top). Scatterplot of length of cardiac arrest versus age (bottom).

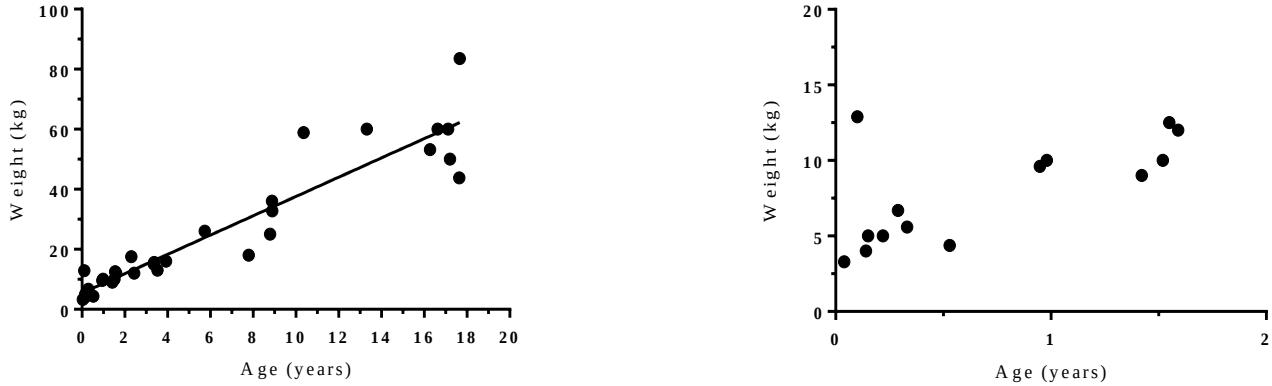


Figure 9: Correlation between age (years) and body weight (kg) of pediatrics.

Figure on the left includes all patients in this study. Figure on the right includes patients <2 years.

3.9.4.2 Full Model Results (Inclusion of Covariates)

The key analysis steps of the backward elimination process for covariate testing are provided in **Table 9**. Model 88 represented the full model with inclusion of all covariates on V_{max}. Each covariate was subsequently removed and the change in OFV was assessed for each analysis run. The final model was reached after 5 rounds of backward elimination, with weight and temperature as the significant covariates on volume.

Table 9: A subset of PopPK models run during final model building of phenytoin.

Round	Run Number	Description	OFV	Comment
1	88	all covariates of interest added	-1797.654	Full covariate model
2	93	remove height on volume	-1797.28	Not significant
3	97	remove age on volume	-1793.076	Not significant
4	104	remove gender on volume	-1786.220	Not significant
5	112	Remove length of cardiac arrest on volume	-1776.309	Not significant
5	110	=104, Remove weight		Significant, cannot delete this covariate
5	111	=104, Remove temperature		Significant, cannot delete this covariate
5	**104_1**	**=104**	**-1704.167**	**Final model**

Final model highlighted in bold.

The final phenytoin population PK model equation for V_{max} is as follows:

Equation 5: $$V_1 = \frac{weight^{\gamma wt1}}{25} * \theta_{V_1} * exp(\eta_{V_1})$$

Equation 6: $\boldsymbol{V_{max0} = \theta_{V_{max0}} * exp(\eta_{Vmax0} + (weight - 25) * wt_2)}$

Equation 7: $\boldsymbol{V = \theta_V * exp\,(\eta_V + (T - 37) * t_1)}$

where V_{max} is the maximum velocity of metabolism; V_1 is the volume of distribution in liters; θ is the fixed effects parameter; η is the random effects parameter; V_{max0} is the time invariant maximum velocity of metabolism at baseline; γwt1 is the weight effect parameter on volume; wt_2 is the weight effect parameter on V_{max}; t is temperature and t_1 is the temperature effect on V_{maxi} parameter.

The parameters of the final phenytoin population PK model are presented in **Table 10**.

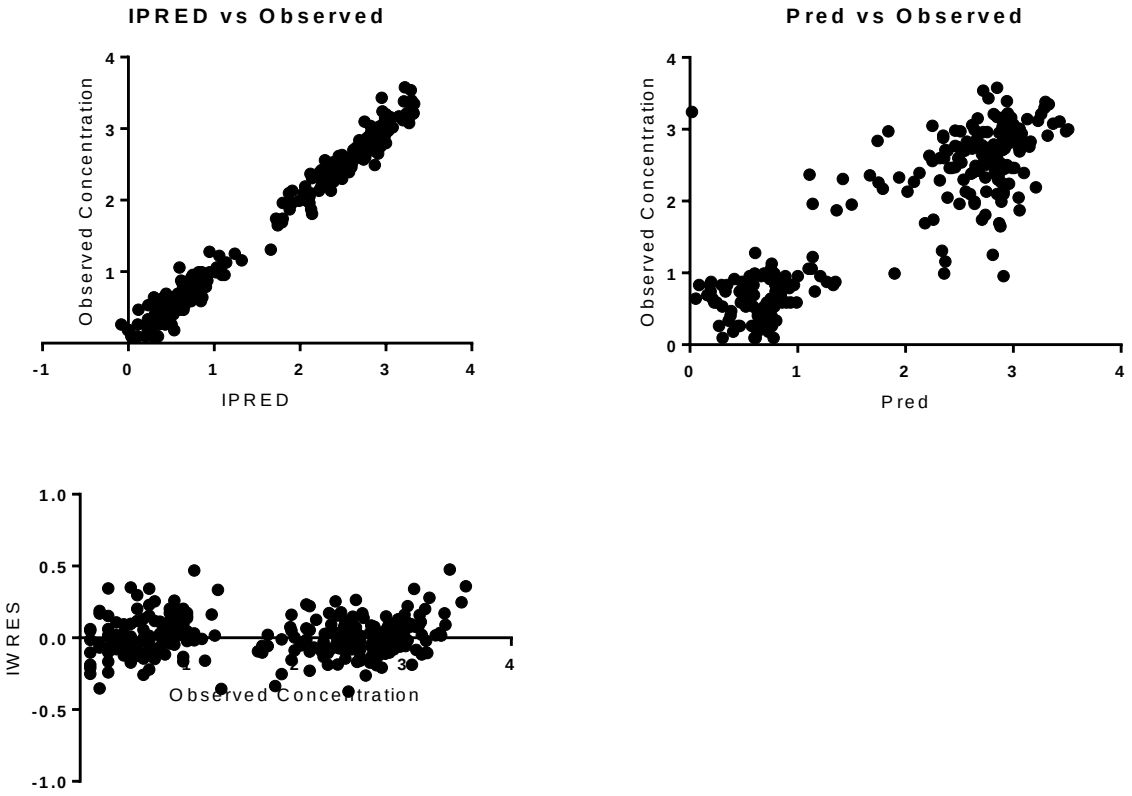
Table 10: Parameter Estimates of Phenytoin Population PK Final Model

Parameter (Units)		
	Point Estimate	**%RSE**
WT	1.05	6.2
WT_2	0.029	4.3
V_1 (L)	360	14.3
V_{max0}	9.17	15.6
k_m (mg/L)	3.17	18.2
V_{max} induced (mg/hr)	12.2	20.5
Inter-individual		
ω^2_{V1}	0.23	12.1
ω^2_{Km}	0 FIX	
Residual variability		
σ^2_{add}	0.0333	13

Footnote: %RSE: percent relative standard error of the estimate = SE/parameter estimate * 100, V_1 = apparent volume of distribution; V_{max0} = time-invariant maximum velocity of metabolism at baseline; WT = weight effect parameter on V_1; WT_2 = weight effect parameter on V_{max0}; V_{maxi} = time-dependent velocity defined by the rate constant k_{ind} and time t; k_m = Michaelis-Menten elimination rate constant.

Figure 10: Goodness-of-fit and diagnostic plots for final phenytoin population PK model.

IPRED: Individual predicted; PRED: Population predicted; IWRES: individual weighted residual.



Circles represent individual data points. The lines represent the local regression (Loess) smoothing lines.

3.9.5 Phenytoin Model Evaluation

3.9.5.1 VPC

A prediction corrected visual predicted check (pcVPC) for the final model of phenytoin data was also analyzed. The pcVPC confirmed that the final phenytoin population PK model provided a good description of the data.

3.9.5.2 Bootstrap Analysis

A bootstrap analysis was performed to compare the estimates generated from 500 simulated subjects to the estimates generated in the NONMEM analysis. Median and 90% CI were comparable between the bootstrap analysis and parameters generated. Appendix B.3. includes comparison between bootstrap and generated PK parameters.

3.9.5.3 Simulations

The final PopPK model of Phenytoin was used to simulate phenytoin concentrations at different degrees of cooling and under clinically relevant conditions. Cooling for 24 or 72 hours demonstrated a decrease in phenytoin elimination, which led to elevated phenytoin concentrations (above the therapeutic range of 2 mg/L).

3.9.5.4 Phenytoin Disposition in Children with CA versus TBI

The current population PK model describing phenytoin disposition in children with CA was compared with the previous PopPK model describing phenytoin PK in children with TBI. Overall, a 1-compartment Michaelis-Menten model best described the PK of phenytoin in both patient populations. Parameters differed between the two populations (V_{max}: 9.17 versus 6.73; K_m 3.17 versus 0.483). Several differences between the two population PK studies may contribute to differences in parameters between patient populations. The previous study in children with TBI consisted of 19 subjects, which is smaller than the current study, however more extensive phenytoin sampling across each subject (121 total and 114 free phenytoin levels) was collected. Importantly, the previous study included both normothermic and hypothermia subjects, unlike the study described here which only includes hypothermic subjects. Additionally, the previous study in pediatric TBI consisted of a hypothermia protocol of 48

hours, as compared to the current study which included hypothermia durations of 24, 48, and 72 hours.

To further compare the PopPK analysis in children with CA versus TBI, the datasets were combined and the final model was re-run (

Table 11). The final population PK model described in Section **3.7** was run on the following four datasets 1) pediatric CA patients only, 2) pediatric TBI patients only (normothermic and hypothermic), 3) critically ill patient dataset comprised of pediatric CA and TBI patients, and 4) compiled pediatric CA patients and normothermic patients from the TBI study.

Overall, the V_{max} and K_m were slightly higher in the pediatric CA patient population (dataset #1) than in the children with TBI population (dataset #2). Interestingly, when both patient populations were combined (dataset #3, "Critically ill patients"), the overall OFV value decreased significantly and the V_{max} and K_m parameters fell between the two patient populations. Further, when the pediatric CA population was compiled with the normothermic patients from the TBI study (dataset #4), a significant decrease in OFV was seen and the V_{max} and K_m parameters decreased slightly from the pediatric CA population alone.

Table 11: Comparison of phenytoin PK parameters from PopPK analysis in children with CA and TBI. A PopPK analysis was run on 4 different datasets to compare final PK parameters across populations.

	Pediatric CA (Ref.)	**Pediatric TBI**	**Critically Ill Pts (TBI + CA)**	**Ped CA Pts and Normotherms from TBI Pt Population**
Dataset #	**1**	**2**	**3**	**4**
ΔOFV from Ref.		+5.2	-423.154	-112.956
WT	1.05	0.809	1.04	1.04
WT_2	0.029	0.0299	0.030	0.034
T_1		0.381	0.0705	0.118
T_2	0 FIX	0 FIX	0 FIX	0 FIX
V_1	360	433	394	370
V_{max}	9.17	6.73	7.17	8.47
K_m	3.17	0.483	1.11	1.54
V_{max} induced	12.2	11.6	12.3	13.1
Omegas				
V_1	0.23	0.0108 (31%)	0.166 (6%)	0.181 (5%)
K_m	0 FIX	0 FIX	0 FIX	0 FIX
V_{max}	0.619 (30%)	0.302 (10%)	0.467 (20%)	0.442 (26%)
Additive Error	0.0333 (13%)	0.0372 (7%)	0.0353 (11%)	0.0421 (12%)

Footnote: OFV = objective function value; WT = weight effect parameter on V_1; WT_2 = weight effect parameter on V_{max0}; T = temperature; V_1 = apparent volume of distribution; V_{maxi} = time-dependent velocity defined by the rate constant k_{ind} and time t; k_m = Michaelis-Menten elimination rate constant; V_{max0} = time-invariant maximum velocity of metabolism at baseline.

3.10 CONCLUSIONS

In summary, a 1-compartment Michaelis-Menten model best described phenytoin levels in pediatrics with CA, which included PK parameters for the elimination rate constant (K_m) and the maximum velocity (V_{max}). This model incorporated a time-varying term for V_{max}, which assumes that the V_{max} of phenytoin is varying over time. Following covariate model building, weight was found to be a significant covariate on the volume of distribution of phenytoin. Temperature was also found to be a significant covariate on phenytoin PK. Collectively, these PopPK results demonstrate that therapeutic hypothermia to 33°C decreased the phenytoin metabolism in this patient population. This decrease in phenytoin metabolism was consistent with findings from a previous PopPK analysis, which demonstrated hypothermia-mediated effects on phenytoin PK in children with TBI. Moreover, simulations indicate that the extent of hypothermia-mediated affects is specific to cooling duration (24 versus 72 hours). Future analysis should investigate these results in a larger patient population, which constitutes both hypothermia and normothermia patients.

3.11 DISCUSSION

The objectives of this study were to characterize the population PK of phenytoin in pediatrics following CA and to identify variability and potential determinants (demographic and clinical covariates) of phenytoin PK during treatment with therapeutic hypothermia. We found that therapeutic hypothermia when applied to pediatrics following a CA decreases the metabolism of phenytoin.

Results of this study are consistent with our previous study (children with TBI), which demonstrated that therapeutic hypothermia decreased phenytoin elimination in children following traumatic brain injury. Despite differences in study population (CA versus TBI) and cooling duration (24, 48, and 72 hrs), an overall decrease in phenytoin elimination was seen during therapeutic hypothermia in both of these studies. Overall, a 1-compartment Michaelis-Menten model best described the PK of phenytoin in both patient populations. Parameters differed between the two populations (V_{max}: 9.17 versus 6.73; K_m 3.17 versus 0.483). Several differences between the two population PK studies may contribute to differences in parameters between patient populations. The previous study in children with TBI consisted of 19 subjects, which is smaller than the current study, however more extensive phenytoin sampling across each subject (121 total and 114 free phenytoin levels) was collected. Importantly, the previous study included both normothermic and hypothermia subjects, unlike the study described here which only includes hypothermic subjects. Additionally, the previous study in pediatric TBI consisted of a hypothermia protocol of 48 hours, as compared to the current study which included hypothermia durations of 24, 48, and 72 hours.

Hypothermia-mediated effects on drug elimination is particularly important with drugs, such as phenytoin, that have a long half-life ($t_{1/2}$ = 20.7 ± 11.6 hours in neonates) (Donovan, Griffin *et al.* 2016). It is important for clinician's to recognize that the effects of therapeutic hypothermia may be seen long past the cooling and rewarming phases into the post-treatment phase when drugs with long half-lives are administered. This creates an extended time period of potentially unexpected drug responses after the active cooling period in which clinicians should continue to monitor drug levels.

In this study, age was not found to be a significant covariate on phenytoin PK. However, previous studies report an effect of age on phenytoin PK due to an age-dependent decrease in the metabolic rate (Battino, Estienne *et al.* 1995). The half-life of phenytoin is generally longer in neonates, of which it decreases following the first postnatal month and then increases with age. In this study, patients were on average 5.7 years old, with 4 patients being less than 2 months, 11 patients between 2 months and 2 years old, 11 patients between 2 and 11 years old, and 6 patients between 11 and 18 years old. The remaining patients in the study did not have a recorded age. While this dataset did not support age as a significant covariate on phenytoin PK, weight was found to be a significant covariate, which was highly correlated with age. One possible explanation that age was not identified as a significant covariate on phenytoin PK is the small number of neonates included in this study.

Other studies have used various compartmental models to describe the population pharmacokinetics of phenytoin (Odani, Hashimoto *et al.* 1996, Ahn, Cloyd *et al.* 2008, Tanaka, Kasai *et al.* 2013). Tanaka *et al.* described phenytoin using a linear 2-compartment model. This study consisted of pooled Phase 1/2 trials following an intravenous administration of fosphenytoin sodium (Tanaka, Kasai *et al.* 2013). This study included numerous plasma samples

following each fosphenytoin dose (n = 923 plasma samples from 24 healthy volunteers) and therefore was able to capture the conversion of fosphenytoin to phenytoin in the model. Given the sparse sampling of phenytoin in our clinical study, the conversion of fosphenytoin to phenytoin was unable to be characterized in our model.

Another study by Odani *et al.* performed a population pharmacokinetic analysis on phenytoin serum concentrations at steady-state (n = 531) from 116 epileptic patients (Odani, Hashimoto *et al.* 1996). This study used a 1-compartment model with Michaelis-Menten elimination to best describe the PK of phenytoin in this patient population. This study reported a V_{max} of 9.80 mg/d/kg and a K_m of 9.19 micrograms/ml. Weight and a co-administered drug, zonisamide, were included in the model. The inclusion of zonisamide was based on an observation during TDM, which indicated higher phenytoin concentrations when co-administered with zonisamide. A number of patients were taking other anticonvulsants including carbamazepine, valproic acid, phenobarbital, and "others". None of these were included in the model. In the present study, we tested the effect of two co-administered medications (levetiracetam and fentanyl) on phenytoin PK. Neither was found to be a significant covariate on phenytoin PK in this population. This is consistent with previous studies which have found no significant DDI between fentanyl and phenytoin. Levetiracetam, which is predominately renally-eliminated was not anticipated to affect phenytoin elimination; however, it's important to note that this analysis included only a small number of patients who were co-administered levetiracetam and phenytoin.

Another study by Ahn *et al.* used a population PK approach to describe the effects of age and sex on phenytoin in adult patients with epilepsy (Ahn, Cloyd *et al.* 2008). In this study, a linear 1-compartment model with a drug depot compartment for fosphenytoin best described the

data. Of the 63 subjects included in this study, there was no difference in the clearance, volume of distribution, or half-life of phenytoin between elderly versus adult subjects nor between male versus females. While this study was conducted in a different population (adults with epilepsy) as compared to the present analysis (pediatrics with CA), we also found no significant difference in phenytoin PK between age or sex.

In all three of the studies described above, phenytoin PK parameters were correlated with body weight in the final model, which is consistent with what we found. A few differences can be seen between the PopPK analysis in our study versus the ones described above. First, the patient populations vary between studies. Tanaka *et al.* included pediatric patients, as well as adult and healthy volunteers. The population studied by Ahn *et al.* and Odani *et al.* both consisted of adult patients with epilepsy. The different patient populations (both disease state and age) and study designs (dosing and sampling frequency/duration) likely explains the difference in PK models which were used to describe phenytoin PK. Additionally, Ahn *et al.* and Tanaka *et al.* consisted of a single IV dose of phenytoin with numerous PK samples whereas our study consists of sparse PK sampling (approximately 7 per subject across 7-9 days).

The main limitations of this study are 1) lack of normothermic subjects; 2) small number of serum samples per subject and 3) lack of free phenytoin levels for samples quantified by UPLC-MS/MS. The lack of a comparator (normothermic) group in this study prevented associations of phenytoin levels with clinical covariates between the two groups. However, given the extensive temperature and sample collection throughout this study, the effect of temperature across different treatment phases (hypothermic, rewarming, and post-treatment) served as an internal control for patients. Additionally, we were able to compare phenytoin PK in pediatrics with CA with our previous analysis in children with TBI, which included a normothermic group.

While disease-mediated affects (cardiac arrest versus traumatic brain injury) are also a possible contributor of phenytoin PK variability, the combined study populations (pediatric CA and TBI) reduced overall variability in the model. Further, disease severity was tested as a covariate in pediatrics with CA (length of cardiac arrest) and in pediatrics with TBI (Glasgow Coma Score and Injury Severity Score), but neither were found to be significant contributors of PK variability in these small sub-populations. Future studies should investigate hypothermia-mediated effects on phenytoin PK in cooled versus non-cooled children following CA.

4.0 EFFECT OF TEMPERATURE ON ABC DRUG TRANSPORT ACTIVITY *IN VITRO*

4.1 INTRODUCTION

As demonstrated in previous Chapters, therapeutic hypothermia has been shown to decrease drug metabolism via CYP450 metabolic pathways leading to an increase in drug concentrations (Tortorici, Mu *et al.* 2009, Hostler, Zhou *et al.* 2010, Zhou, Empey *et al.* 2011, Empey, Miller *et al.* 2012). To date, pharmacokinetic studies investigating the effects of therapeutic hypothermia on drug disposition are almost exclusively focused on hepatic drug metabolism. Evidence is limited supporting other important components of pharmacokinetics such as drug absorption and distribution. Specifically, drug transporters play a role in drug distribution and pharmacokinetics. Of the multitude of drugs administered to critically ill patients, many of them undergo transporter pathways in addition to metabolic pathways (**Table 12**). Despite this, there is minimal evidence investigating clinically relevant temperature-mediated effects on drug transporters. The following sections describe the important role of drug transporters in drug disposition, the current evidence of hypothermia-mediated effects on drug transport, and the aims and hypothesis of the studies described in this Chapter.

Table 12: Drugs administered in the ICU and their transporter pathways.

Drug	**Transporter(s) Pathway**
Antiarrythmics	
Digoxin	ABCB1
Quinidine	ABCB1
Antibiotics	
Ciprofloxacin	SLC22A8
Antihypertensives	
Enalapril	ABCG2, SLCOs
Prazosin	ABCG2
Valsartan	ABCG2, SLCOs
Statins	ABCG2, SLCOs
Opioids	ABCB1
Miscellaneous	
Cimetidine	ABCG2
Dipyradamole	ABCG2
Vecuronium	ABCB1
Protease Inhibitors	ABCG2, ABCB1
Fluoroquinolones	ABCG2
Verapamil	ABCB1
Diltiazem	ABCB1
Phenytoin	ABCB1
Vecuronium	ABCB1

4.2 ROLE OF DRUG TRANSPORTERS IN DRUG DISPOSITION

Drug transporters have been increasingly recognized for their important role in drug pharmacokinetics (Schinkel and Jonker 2003, Sai 2005, 2010, Tweedie, Polli *et al.* 2013). To date, a number of studies have demonstrated that transporters, along with drug metabolizing enzymes, play a role in therapeutic efficacy, drug safety, and ADEs (2008, Huang, Strong *et al.* 2008, 2010). Specifically, two main transporter superfamilies, the solute carrier (SLC) and ATP-binding cassette (ABC), account for over 400 transporters that are widely distributed throughout the body (Giacomini, Huang *et al.* 2010). Clinically observed drug-drug interactions with drug transporters have been reported for a number of drugs (König, Müller *et al.* 2013) such as inhibition of P-gp mediated digoxin transport by quinidine (Ochs, Bodem *et al.* 1981, Schenck-Gustafsson and Dahlqvist 1981, Fenster, Comess *et al.* 1982, Fenster, Hager *et al.* 1984); inhibition of OCT2 and MATE-mediated transport of methotrexate by probenecid (Aherne 1978); and inhibition of BCRP-mediated transport of elacridar by topotecan (Kruijtzer, Beijnen *et al.* 2002), to name a few. However, as was previously discussed in Chapter 1 (**1.2.1**), drug-therapy interactions do not undergo the same degree of testing as drug-drug interactions. Consequently, little is understood as to the role of drug transporters during drug-therapy interactions, such as therapeutic hypothermia. The subsequent section describes the current *in vitro* evidence that has investigated therapeutic hypothermia on drug transport to date.

4.3 EVIDENCE THAT THERAPEUTIC HYPOTHERMIA EFFECTS ABCB1 DRUG TRANSPORT ACTIVITY

To date, only one study has investigated therapeutic hypothermia on drug transporter activity. This study investigated the effect of therapeutic hypothermia on one of the ABC drug transporters, ABCB1 (P-gp). Jin *et al.* reported a decrease in active transport of ABCB1 during cooling, but no change was seen in passive diffusion (Jin, Sakaeda *et al.* 2006). In this study, LLC-PK_1 cell lines transfected with human MDR1 and their respective wild-type cells were treated with 3 hours of hypothermia (32˚C) or normothermia (37˚C). Two radiolabeled probes were used to measure ABCB1 transport across cell membranes, [^{3}H]digoxin and [^{3}H]quinidine. The results demonstrate that active ABCB1 transport of the radiolabeled probe substrate [^{3}H]digoxin was decreased by 50% under mild hypothermic conditions (32˚C). The transport of [^{3}H]digoxin continued to significantly decrease below therapeutic hypothermia temperatures down to as low as 4˚C. In addition, the effects of cooling on the other ABCB1 probe substrate, [^{3}H]quinidine, was also investigated in this cell line. Interestingly, quinidine transport only decreased at a temperature of 4˚C, but not at temperatures of 32°C or 25°C. The authors suggest that a possible explanation for the selective effect of cooling on [^{3}H]digoxin versus [^{3}H]quinidine transport could be due to the fact that quinidine is also a substrate for the organic cation transporter (OCT), which is expressed in the LLC-PK1 cell line used in this study.

Additionally, Jin *et al.* measured paracellular transport using a specific radiolabeled marker, [methoxy-^{14}C]inulin. No effect of cooling was seen on paracellular transport down to 25°C. Further, [^{3}H]tetracycline, a non-Pgp substrate, was used as a control. Overall, the net transport (B to A/A to B) was not altered at lower temperatures, however the unidirectional transport (B to A and A to B) was decreased at lower temperatures in both directions. The

authors suggest that this may be due to a change in transcellular transport mediated by passive diffusion.

Results of this study demonstrate an effect of therapeutic hypothermia on ABCB1 drug transport by as much as 50% *in vitro*. In addition to ABCB1, other drug transporters play an important role in drug disposition, of which the impact of temperature-mediated changes is largely unknown. Therefore, we aimed to expand on what's currently known to include additional ABC drug transporters. Additionally, we aimed to focus on clinically meaningful temperatures to investigate how drug transporters may be affected under different temperatures (including both hyper- and hypo- thermic conditions).

4.4 AIMS AND HYPOTHESIS OF STUDY

We aimed to investigate the effects of clinically relevant temperatures (therapeutic hypothermia and hyperthermia) on the active drug transport of three ABC transporters: ABCB1, ABCG2, and ABCC1. We hypothesize that therapeutic hypothermia will lead to a decrease in the active drug transport of all three ABC drug transporters, while having no effect on the passive transport across cell membranes. Further, we anticipate that the magnitude of these effects will likely be different between transporters since transporter function is dependent on specific drug-transport interactions.

4.5 METHODS

4.5.1 Materials and Chemicals

[^{3}H]digoxin and [^{14}C]sucrose were purchased from PerkinElmer (Waltham, MA). [^{3}H]cimetidine and [^{3}H]estradiol-17-β-D-glucuronide were purchased from American Radiolabeled Chemicals (St. Louis, MO). Gibco MEM Optimem cell culture media was purchased from Invitrogen (#41090-101). Additional reagents were purchased from the following: FBS, Optima, heat-inactivated (Atlanta Biologicals #S12450H), Pen/strep (Fisher (Cellgro) #MT-30-002-C), Genecitin (Fisher (Cellgro) #MT-30-234-C).

4.5.2 Cell Lines

The Madin-Darby canine kidney-II (MDCKII)-ABCG2 cell line was developed in the laboratory of Dr. McNamara (University of Kentucky, Lexington, Kentucky) (Wang, Leggas *et al.* 2012). The MDCKII-ABCB1 and ABCC1 cell lines were obtained from Dr Piet Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). MDCKII-ABCG2 cells were cultured in Gibco Minimum Essential Media (MEM), Earle's salts, with glutamax (Invitrogen #41090-101) with 5% FBS, Optima,, heat-inactivated (Atlanta Biologicals #S12450H) and 800 ng/mL genecitin (Fisher(Cellgro) #MT-30-234-C). MDCKII-ABCB1 cells were cultured in Gibco Minimum Essential Media (MEM), Earle's salts, with glutamax (Invitrogen #41090-101) with 10% FBS and 800 ng/mL genecitin. MDCKII-ABCC1 cells were cultured in DMEM, High Glucose, GlutaMAX™ (Invitrogen # 10566024) with 1mM Sodium Pyruvate (Invitrogen # 11360070 Lot# 1227460), 10% FBS Optima, heat-inactivated (Atlanta Biologicals #S12450H) and 1%

Pen/strep (Fisher(Cellgro) #MT-30-002-C). All cell lines were grown in incubators at 37°C in a humidified atmosphere of 5% CO_2.

4.5.3 Transepithelial Drug Transport Assay

Cells were initially grown at a density of 2×10^6 cells per 24.5 mm well on transwell polycarbonate filters with a 3.0 μm pore size (Corning Inc., Corning, NY). Cells were seeded onto polycarbonate filters to form monolayers.

The MDCK-II cell line is a polarized cell line derived from the kidney of canines. When grown to form monolayers, the apical membranes face up while the basal membranes are attached to the microporous polycarbonate filter. In MDCKII cells overexpressing-ABCB1, ABCB1 is highly expressed on the apical membrane and acts as an efflux transporter. Thus, transport from the basal to the apical side (B→A) is increased and transport from the apical to the basal side (A→B) is decreased for specific ABCB1 probes in the over-expressed cell line as compared to the empty (control) cell line (**Figure 11**). Therefore, ABCB1-mediated transport can be assessed in these cell lines using specific probe substrates. Similarly, ABCG2 and ABCC1 can be assessed in the same fashion in MDCKII-overexpressed versus control cells.

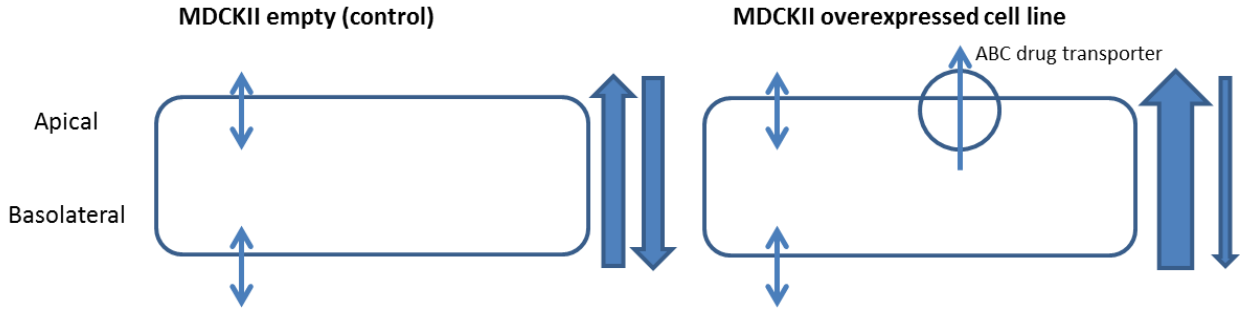


Figure 11: Illustration of drug transporter expression across cell membranes.

Following seeding to transwells, cells were cultured for 4-6 days with media changed once every two to three days. To confirm the quality of cell monolayers, transepithelial electrical resistance (TEER) was measured with an epithelial voltohmmeter (World Precision Instruments, Sarasota, FL). Cell monolayers with TEER values lower than 100 Ω were not used for flux assay. Immediately prior to the start of experiment, old media was removed, and cells were washed once and then replaced with OptiMEM (2 mL in each basolateral and apical side of transwell). The transport across the cell monolayer was conducted by adding the following specific transporter probes to each of the over-expressed cell line: [^{3}H]cimetidine for ABCG2 cell line; [^{3}H]digoxin for ABCB1 cell line; [^{3}H]estradiol-17-β-D-glucuronide for ABCC1 cell line. In addition, [^{14}C]sucrose was added with probes to assess the degree of tight junction formation. Probes and sucrose were added to either the apical or basolateral side of the monolayer to measure transport in both directions across the member (basal to apical, B→A and apical to basal, A→B). Transwells were stored in incubators until sampling. Samples (50 μL) were taken from the opposite side of the cell monolayer at 0, 30, 60, 120 and 240 minutes. Radioactivity was then measured using a liquid scintillation counter.

4.5.4 Apparent Permeability and Efflux Ratio Calculations

Following radioactivity measurements, the observed permeability (P_{app}) of probe substrates or paracellular (sucrose) marker were determined by calculating its initial transfer rate across the cell monolayer and dividing by the surface area of the membrane filter and the initial concentration in the donor chamber (**Equation 8**) where dQ/d_t is the permeability rate of the probe substrate, A is the surface area of the monolayer and C_o is the initial concentration in the

donor compartment. The overall flux of each probe substrate was determined by best fit line through the linear region of the graph of the cumulative pmol transferred versus time. Linear regression was performed using GraphPad Prism version 4.03 (San Diego, CA). A paracellular leakage of less than 1% per hour is considered negligible (Pavek, Merino *et al.* 2005). Therefore, monolayer integrity was considered acceptable and flux was calculated as long as paracellular marker did not exceed 1% per hour.

Equation 8: $$\boldsymbol{P_{app} = \frac{1}{A * C_o} * \left(\frac{dQ}{dt}\right)}$$

The efflux ratio (ER_α) was calculated by dividing the transepithelial drug efflux rate of (B→A) by (A→B) to evaluate drug transporter-mediated directional efflux (**Equation 9**). Data was expressed as mean ± S.D.

Equation 9: $$\boldsymbol{ER_\alpha = \frac{Papp_{B\rightarrow A}}{Papp_{A\rightarrow B}}}$$

4.5.5 Statistical Analysis

Data are expressed as mean ± S.D. from two to three observations for transwell assays. Statistical significance was evaluated by the Student's t-test (unpaired, two-tailed, $\alpha = 0.05$) and by a two-way ANOVA followed by the Bonferroni post-t-test ($\alpha = 0.05$). All statistical analyses were performed using GraphPad Prism version 4.03 (San Diego, CA) with a p-value<0.05 considered significant.

4.6 CONTROL EXPERIMENTS

4.6.1 Temperature Control Experiments

It is critical in these studies that we achieve and maintain consistent temperatures throughout these experiments in order to accurately quantify a temperature effect and eliminate any potential noise in this data. Therefore, we began by conducting a series of control experiments to measure the temperature within 1) cell culture media prior to distributing into transwells, 2) incubators, and 3) cell culture media post dispensing into transwells.

4.6.1.1 Temperature of Cell Culture Media Prior to Distribution to Transwells

At the start of flux experiments, the cell culture media in transwells is rinsed and replaced with OptiMEM. The OptiMEM is stored in a refrigerator until time of experiment. Prior to experiments, OptiMEM is warmed in a water bath (set to 37°C) and then dispensed into transwells. In this control experiment, we aimed to record the temperature of OptiMEM while in the water bath in order to determine the optimal pre-incubation time necessary to achieve target temperature. A mercury thermometer was used to measure the temperature of the OptiMEM and ensure it achieved appropriate temperature before it was distributed into transwell plates.

During experiment, OptiMEM was removed from the fridge and placed in a 37°C water bath. Temperature was measured over 15 minute increments up until the desired temperature was achieved in the media (**Figure 12**). After 2 hours in the water bath, the OptiMEM achieved close to the desired temperature (36.5°C). Next, we removed the OptiMEM from the water bath, transferred to the hood, and measured how quickly the temperature decreased. After 15 minutes, the temperature of the OptiMEM was at 31.5°C. Based on these results and the highly variable

and quick changes in temperature, we conducted a series of additional control experiments (subsequent sections) to further determine how to maintain consistent and accurate temperatures throughout experiments.

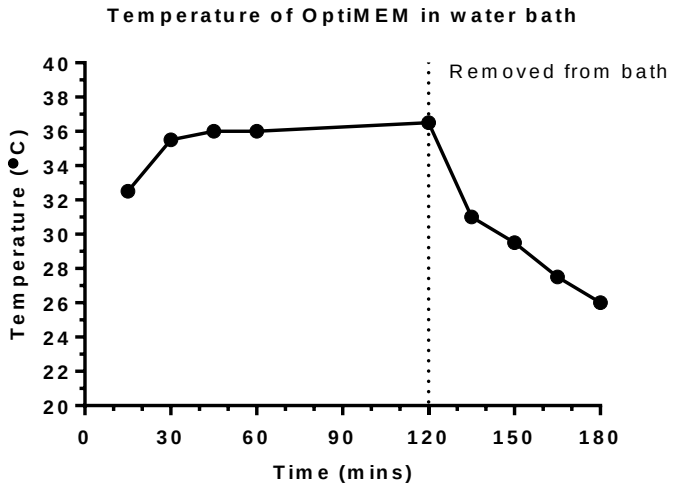


Figure 12: Temperature of cell culture media when warmed in water bath prior to experiment.

4.6.1.2 Temperature within Incubators

The objective of this control experiment was to determine the temperature within incubators by measuring 1) how consistent incubators maintained temperature and 2) recovery time of temperature in incubators under experimental conditions. Incubators were set to 40°C, 37°C, 33°C and 30°C. **Figure 13** and **Figure 14** depict the temperature of the incubators set to 37°C and 33°C, respectively, during simulated experimental conditions. Sharp drops in temperature can be seen when the incubator door was open. Based on incubator output, incubators take ~1-2 minutes to achieve target temperature and ~5-8 minutes to completely restore to set temperature after the door is open and closed. Therefore, we concluded that 1) the incubators maintain consistent

temperature under experimental conditions over the duration of the study and 2) are able to recover quickly from drops in temperature when the door is open and closed.

In addition to temperature, the stability of the atmospheric pressure inside the incubators was also evaluated. During a simulated experiment, incubators take approximately 2-3 minutes to restore the $\%CO_2$ to set level (5.0%) after the door is open and closed (**Figure 15**). Therefore, we concluded that 1) atmospheric pressure in the incubators was consistent throughout the study and 2) atmospheric pressure recovered quickly from changes under experimental conditions.

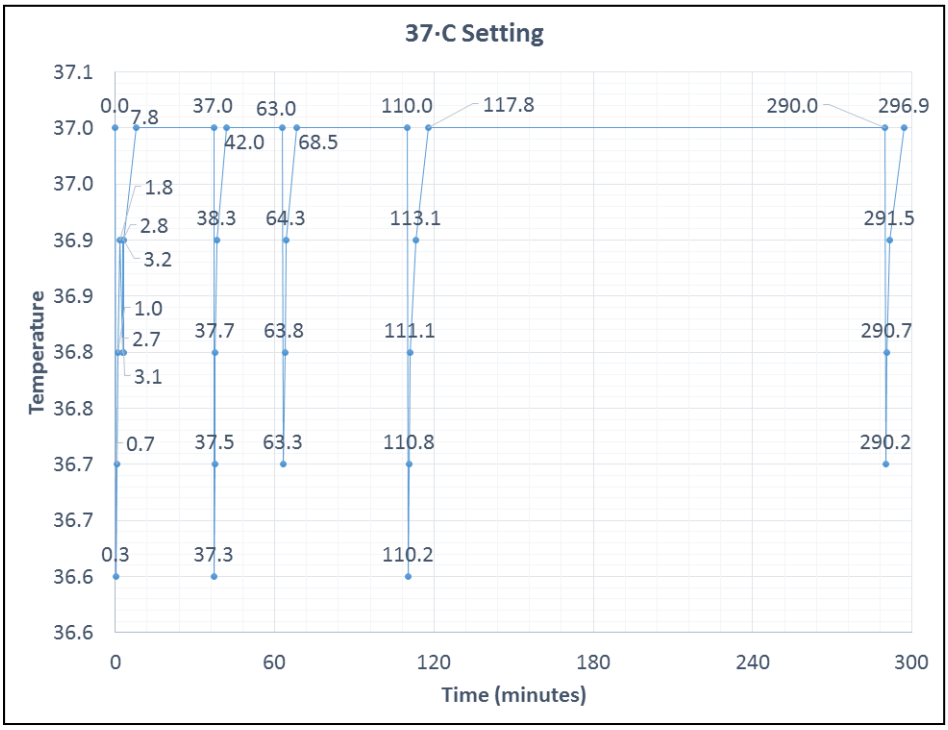


Figure 13: Temperature stability of incubators (37°C).

The incubator door was open at the following sampling timepoints: 0, 30, 60, 120, and 290 minutes.

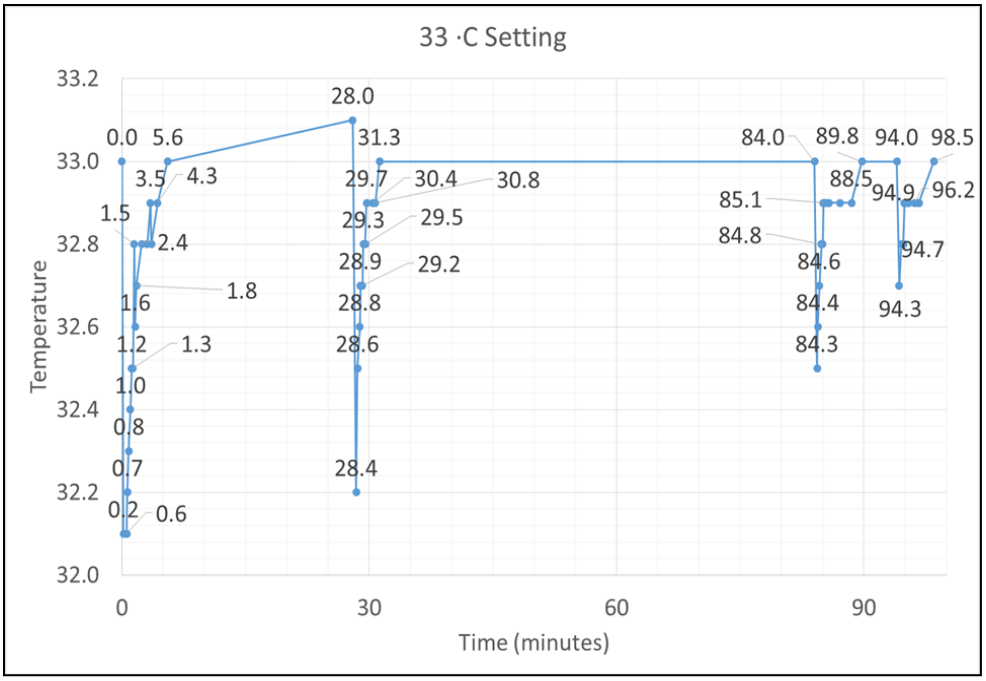


Figure 14: Temperature stability of incubators (33°C).

The incubator door was open prior to the following sampling timepoints: 0, 30, 85 and 95 minutes.

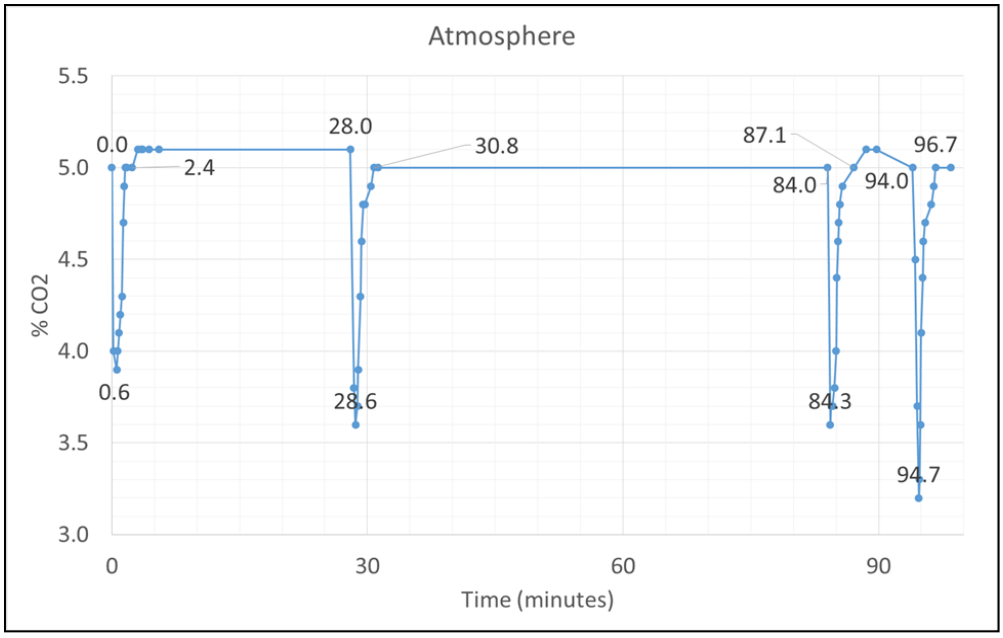


Figure 15: Atmospheric stability of incubators (5.0% CO_2).

The incubator door was open prior to the following time points: 0, 30, 85 and 95 minutes.

4.6.1.3 Temperature of Media within Transwells

Based on the output from the incubator study, we concluded that temperature was achieved and maintained in the incubators throughout the duration of the study. However, the results of the OptiMEM study demonstrated sharp declines in temperature once removed from the water bath. Therefore, the next question we asked was if the target temperature was achieved in the transwells and maintained during the study duration. To answer this question we used a temperature probe, which was inserted into the media in the transwell, and recorded the temperature reading during a simulated experiment. The following describes the results of this study.

In these experiments, incubators were set to 40°C, 37°C, 33°C and 30°C. Temperature was recorded from the incubator output and from the probe within the media in the transwell. **Figure 16** depicts the incubator and probe temperatures over the course of the experiment when

the incubator was set to 33°C. Incubator temperature was within 33 ± 0.7 °C while transwell temperature ranged from 28.6 - 33.2°C and was within 33±1°C during 35.4% of the experiment. **Figure 17** depicts the incubator and probe temperatures over the course of the experiment when the incubator was set to 37°C. Incubator temperature was within 37 ± 0.8 °C while transwell temperature ranged from 31.8 – 36.5°C and was within 37±1°C during 31.2% of the experiment. **Figure 18** depicts the incubator and probe temperatures over the course of the experiment when the incubator was set to 40°C. Incubator temperature was within 40 ± 1.0 °C while transwell temperature ranged from 31.5 - 39.6°C and was within 40±1°C during 47.9% of the experiment. Based on these results, we concluded that temperature was highly inconsistent between the incubator temperature and the media temperature and highly variable throughout the study.

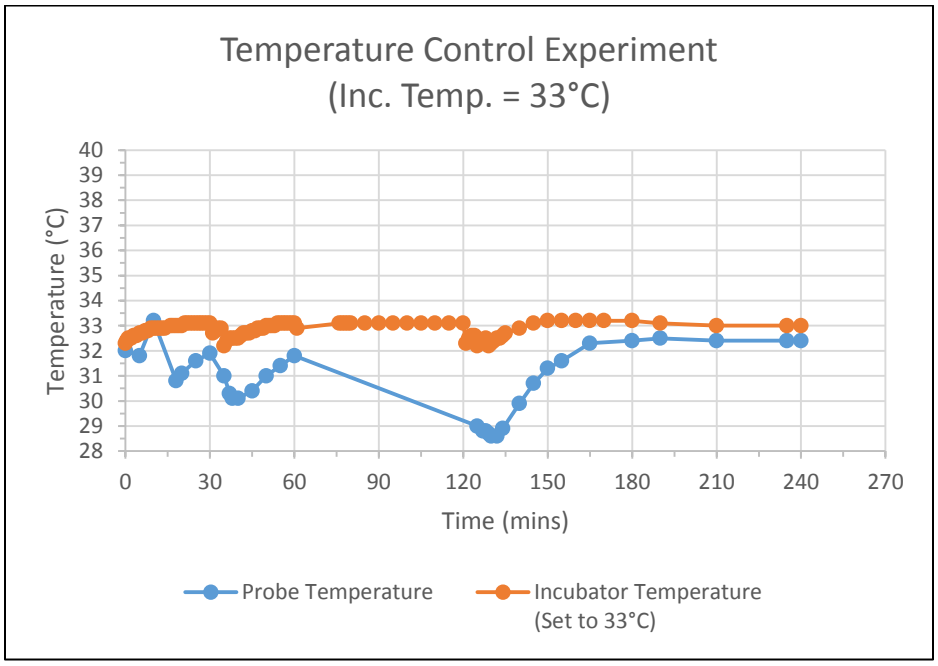


Figure 16: Temperature within transwells (33°C).

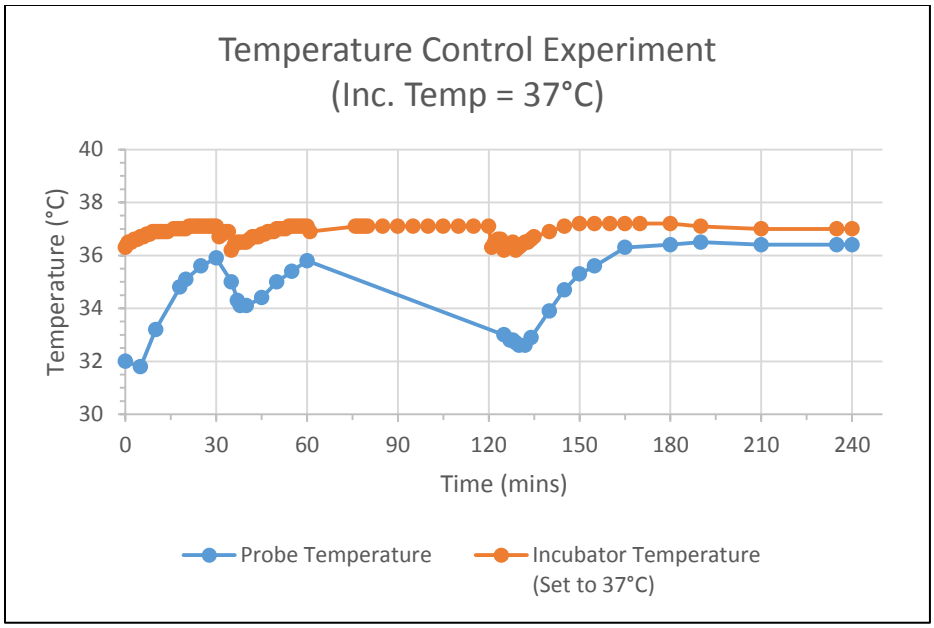


Figure 17: Temperature within transwells (37°C).

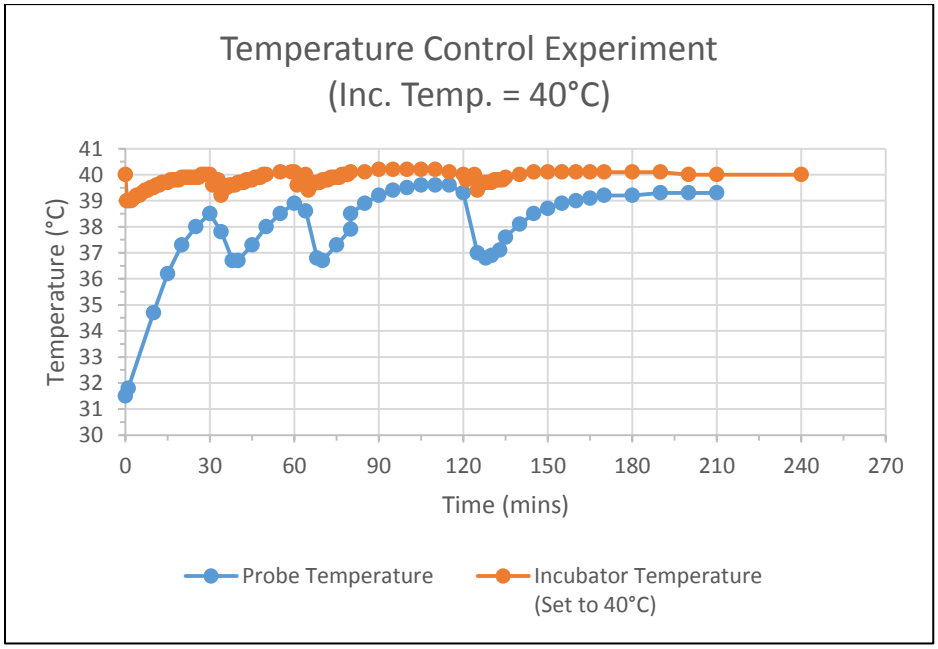


Figure 18: Temperature within transwells (40°C).

To address these temperature discrepancies, we added a heating pad inside the cell culture hood. Transwell plates were transferred directly from incubators into the hood and placed on the heating pad to preserve temperature. This prevented significant loss of heat during sampling time in the hood. Additionally, the length of time to warm the media was extended in order to ensure that media reached desired temperature before being dispensed into transwells. Further, aliquots of OptiMEM were removed from the bottle to achieve target temperature faster. Additionally, aliquots of OptiMEM were transferred from the water bath to a beaker of water in the hood on a hot plate. This helped to preserve the temperature of OptiMEM while being transferred to transwells. Temperature control experiments were repeated under revised conditions and results are discussed in subsequent section (**4.6.1.4**).

4.6.1.4 Revised Methods to Maintain Temperature in Transwells

Temperature experiments were repeated, as described in previous section, under revised experimental conditions with the intent of achieving more consistent and accurate target temperatures during the course of the experiment. When incubator was set to 30°C, transwell temperature ranged from 29.4 - 30.3°C and was within 30.0±0.9°C for 100% of the experiment (**Figure 19**). When incubator was set to 33°C, transwell temperature ranged from 32.0 - 33.2°C and was within 33.0±1.0°C during 100% of the experiment (**Figure 20**). When incubator was set to 37°C, transwell temperature ranged from 35.3 - 37.1°C and was within 37.0±1.0°C during 95.8% of the experiment (**Figure 21**). When incubator was set to 40°C, transwell temperature ranged from 35.6 - 39.4°C and was within 40.0±1.0°C during 47.1% of the experiment (**Figure 22**). Results demonstrated that with the revised heating methods, temperature was consistent

during the study duration. Therefore, we concluded that temperature-mediated effects could be accurately quantified under experimental conditions.

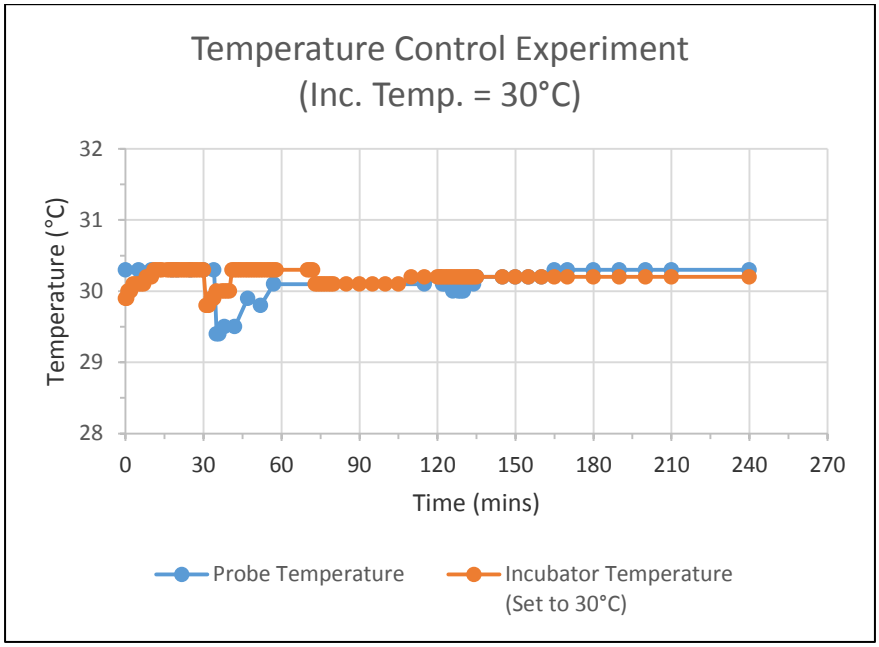


Figure 19: Temperature of media versus incubator temperature (30°C).

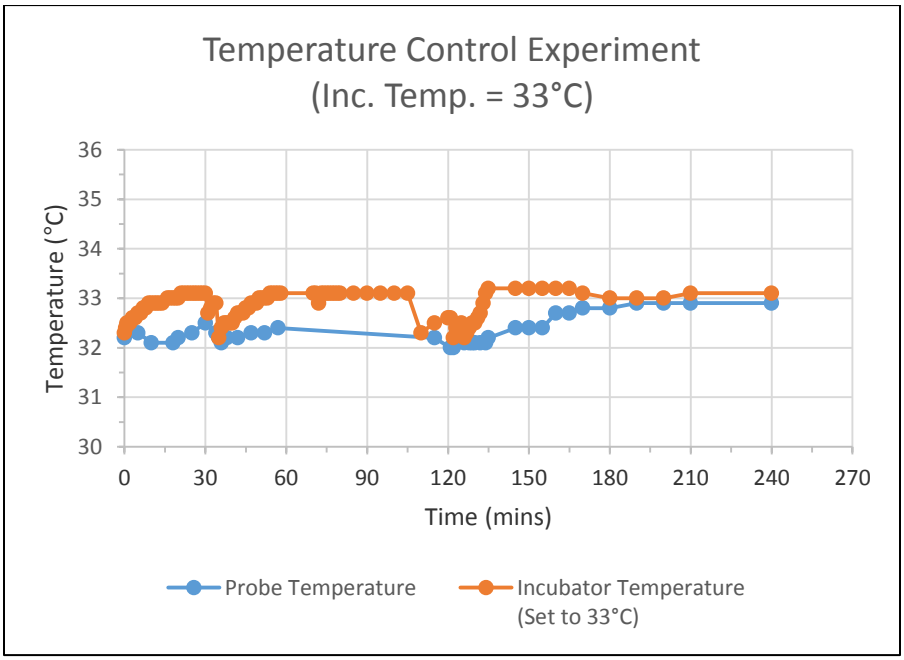


Figure 20: Temperature of media versus incubator temperature (33°C).

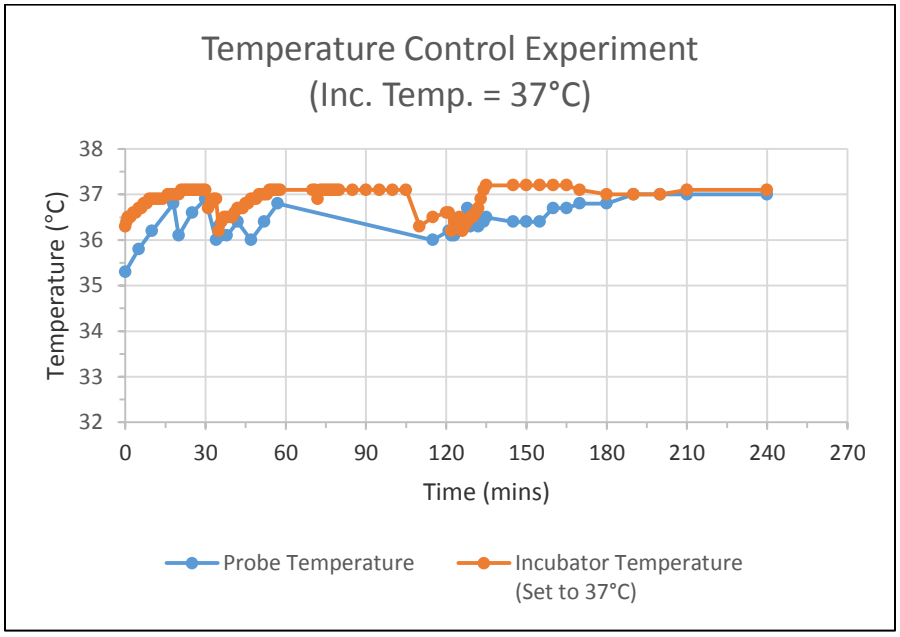


Figure 21: Temperature of media versus incubator temperature (37°C).

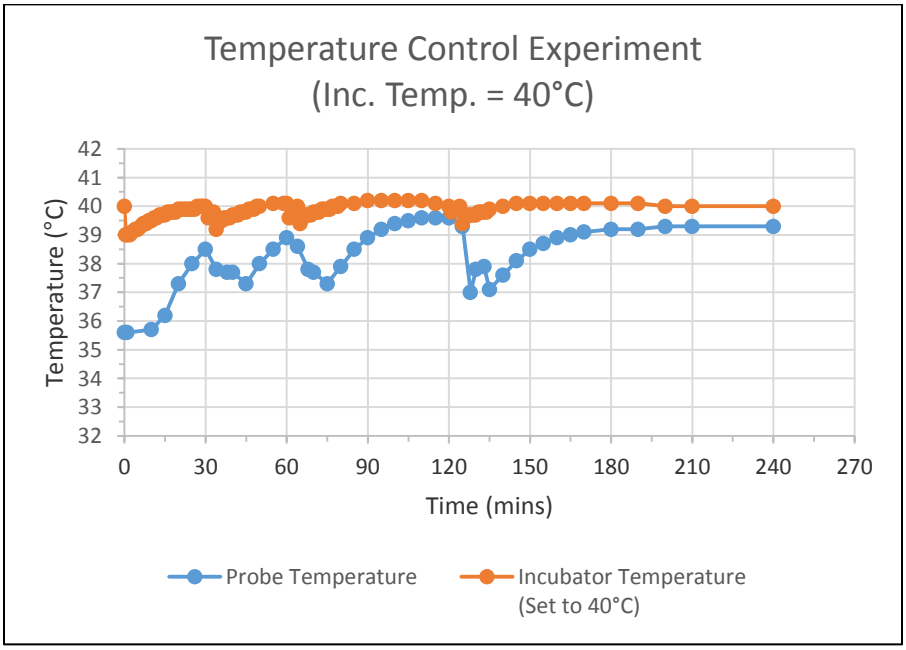


Figure 22: Temperature of media versus incubator temperature (40°C).

4.6.2 Concentration Control Experiments

After controlling for the temperature throughout the study, we next aimed to conduct concentration assays in order to determine the linearity of transport for each probe and their drug transporter. Concentration assays were conducted to select the probe concentration to use in permeability flux assays. The following sections describe the results of the probe concentration versus flux for ABCB1-, ABCG2-, and ABCC1- overexpressed cells.

4.6.2.1 ABCB1-Overexpressed Cell Line

[^{3}H]digoxin, an established probe substrate for ABCB1, was selected for flux assays. The flux of [^{3}H]digoxin (pmol transferred) across the over-expressed cell monolayers was measured at 1, 2.5, 5, and 10 μM. Results demonstrated linear flux of digoxin from 1-10 μM (**Figure 23**). Based on these results, the concentration of drug added to the donor compartment for permeability flux assays was selected to be 5.0 μM.

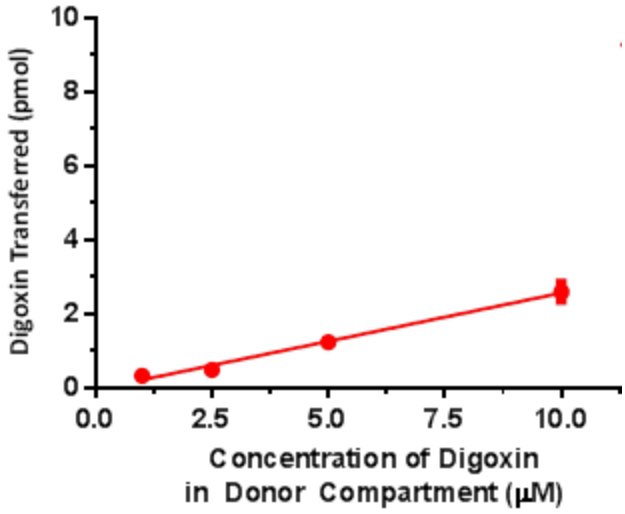


Figure 23: Flux of digoxin across ABCB1-overexpressed cell monolayers.

4.6.2.2 ABCG2-Overexpressed Cell Line

[^{3}H]cimetidine, an established probe substrate for ABCG2, was selected for flux assays. The flux of [^{3}H]cimetidine at 1, 2.5, 5, and 10 μM was measured across ABCG2-overexpressed cell monolayers. Cimetidine flux was linear across ABCG2-overexpressed cell monolayers from 1-10 μM (**Figure 24**). The concentration of drug added to donor compartment in permeability flux assays was selected to be 5.0 μM.

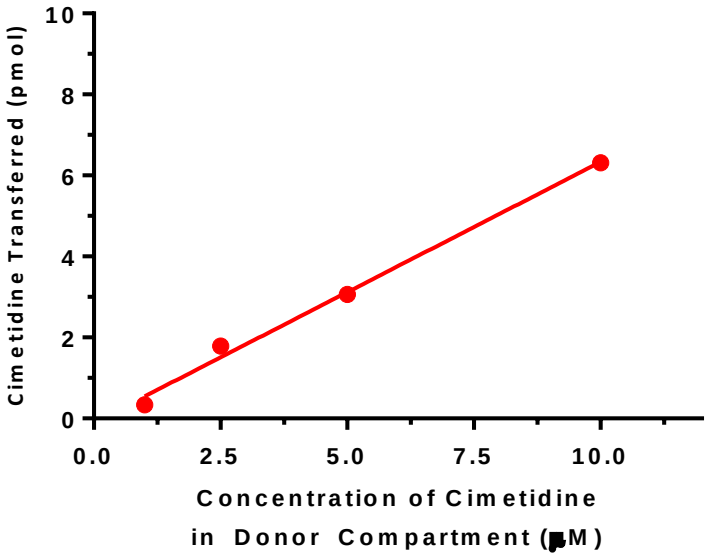


Figure 24: Flux of cimetidine across ABCG2-overexpressed cell monolayers.

4.6.2.3 ABCC1-Overexpressed Cell Line

[^{3}H]estradiol-17-β-D-glucuronide, an established probe for ABCC1, was selected as the probe substrate. The flux of [^{3}H]estradiol-17-β-D-glucuronide at 1, 2.5, 5, and 10 μM concentration was measured. Flux was linear from 1-10 μM (**Figure 25**). The concentration of drug added to donor compartment was selected to be 5.0 μM.

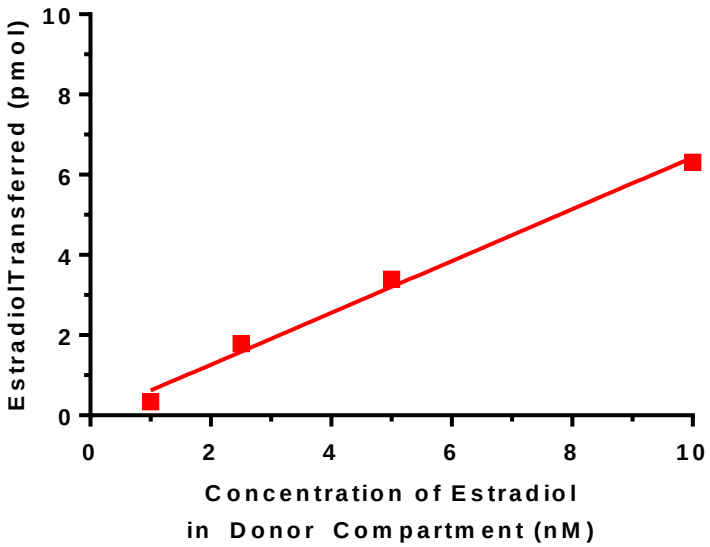


Figure 25: Flux of estradiol-17-β-D-glucuronide concentration across ABCC1-overexpressed cell monolayers.

4.6.3 Time Course Experiments

A time course study was performed to measure the transepithelial transport of [^{3}H]digoxin, [^{3}H]cimetidine, and [^{3}H]estradiol-17-β-D-glucuronide in ABCB1-, ABCG2-, and ABCC1-overexpressing cell monolayers, respectively. The following sections describe the results of these time course experiments in each cell line.

4.6.3.1 ABCB1-Overexpressed Cell Line

The transepithelial transport of [^{3}H]digoxin across the cell monolayers of ABCB1-overexpressing cells was time dependent from 0.5 – 6 hours at 37°C (**Figure 26**), confirming the linearity of digoxin flux up to six hours. Each point represents the mean ± standard deviation from three separate experiments. Based on these results, we selected a 4 hour study duration to perform permeability flux assays.

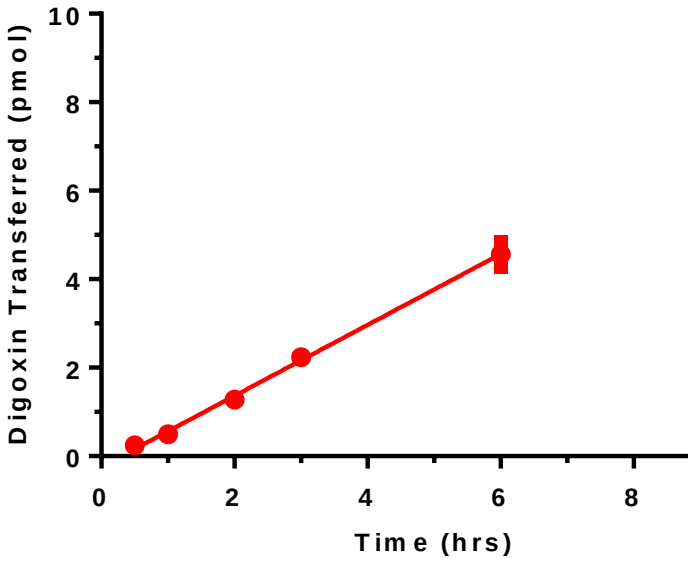


Figure 26: Transepithelial transport of digoxin in ABCB1-overexpressing cells.

Transport was linear from 0.5 – 6 hours at 37°C. Each point represents mean ± standard deviation from three separate experiments.

4.6.3.2 ABCG2-Overexpressed Cell Line

The transepithelial transport of [^{3}H]cimetidine across the cell monolayers of ABCB1-overexpressing cells was time dependent at 37°C and 33°C (**Figure 27**). Each point represents the mean ± standard deviation from three separate experiments. Based on these results which demonstrate the linearity of cimetidine flux up to six hours, we selected a time duration of 4 hours to conduct flux assays.

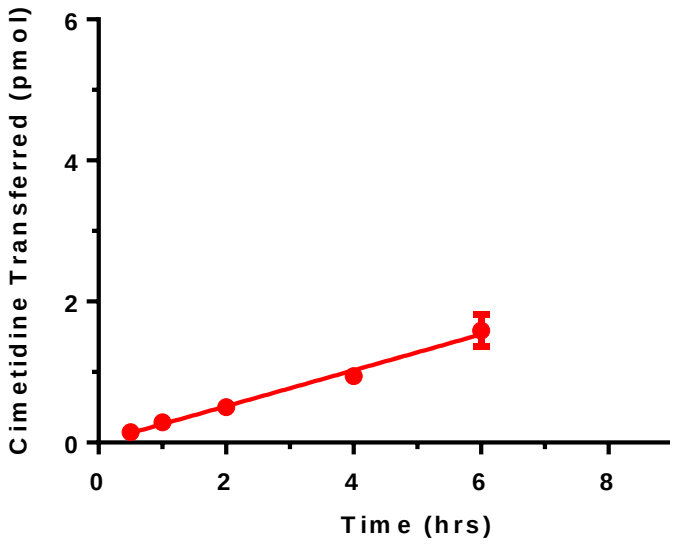


Figure 27: Transepithelial transport of cimetidine in ABCG2-overexpressing cells.

Transport was linear from 0.5 – 6 hours at 37°C. Each point represents mean ± standard deviation from three separate experiments.

4.6.3.3 ABCC1-Overexpressed Cell Line

The transepithelial transport of [^{3}H]estradiol-17-β-D-glucuronide across the cell monolayers of ABCC1-overexpressing cells was time dependent at 37°C (**Figure 28**). Each point represents the mean ± standard deviation from three separate experiments. Based on these results and the linearity of estradiol-17-β-D-glucuronide flux up to six hours, we selected a time duration of 4 hours to conduct flux assays.

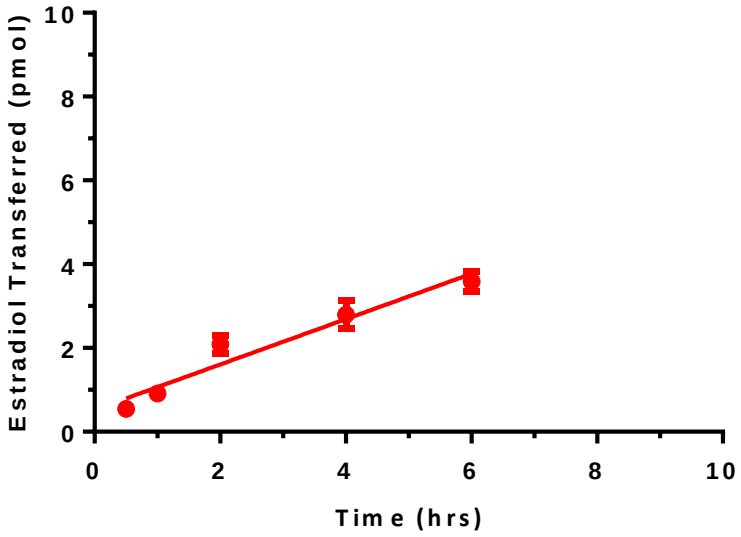


Figure 28: Transepithelial transport of estradiol in ABCC1-overexpressing cells.

Transport was linear from 0.5 – 6 hours at 37°C. Each point represents mean ± standard deviation from three separate experiments.

4.6.4 Confirmation of Overexpressed Cell Lines

In these studies, the flux of [^{3}H]digoxin, [^{3}H]cimetidine, and [^{3}H]e estradiol-17-β-D-glucuronide was measured in ABCB1-, ABCG2-, and ABCC1- overexpressing cell monolayers, respectively, and their respective wild-type cell lines. Flux in both the apical to basolateral and basolateral to apical direction across cell membranes was measured. The transport of probe drug across both directions of the cell monolayer was compared between each over-expressed cell line and their respective wildtype cell line to confirm over-expression of drug transporter. The following sections describe the results of these experiments in each cell line.

4.6.4.1 ABCB1-Overexpressed Cell Line

The basal to apical transport of [^{3}H]digoxin across ABCB1-overexpressed cells was significantly higher than the apical to basolateral transfer. Further, the basal to apical transport was higher and the apical to basolateral transport was lower in the ABCB1-overexpressed cells compared to empty cells (**Figure 29**). This confirms the over-expression of the ABCB1-overexpressed cell line and ensures that it can be used to measure flux.

One interesting observation with the ABCB1-overexpressed cell line is that the apical to basolateral flux was similar to the flux observed in the wildtype cell line. Previous studies also report seeing a higher flux of digoxin in wildtype cells due to endogenous ABCB1 (Kuteykin-Teplyakov, Luna-Tortos *et al.* 2010).

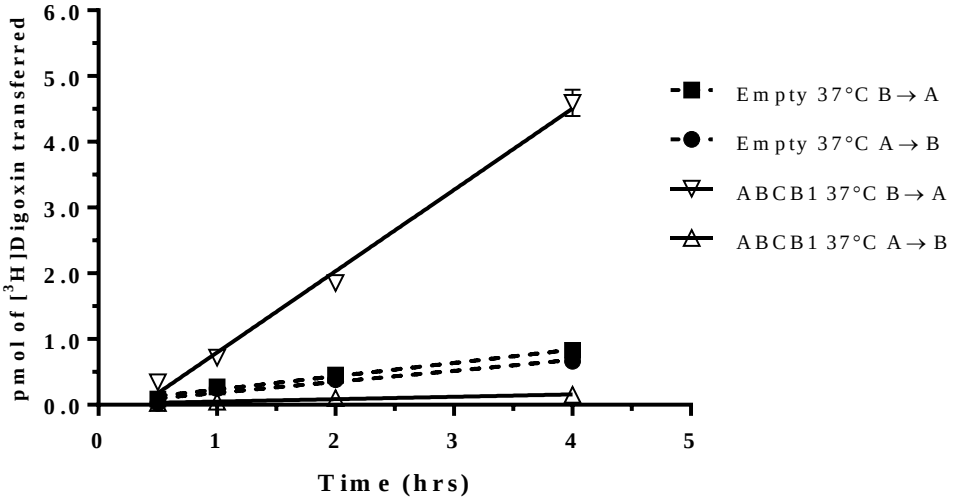


Figure 29: Flux of digoxin across ABCB1-overexpressed and empty- MDCKII cells.

4.6.4.2 ABCG2-Overexpressed Cell Line

The basal to apical transport of [^{3}H]cimetidine across ABCG2-overexpressed cells was significantly higher than the apical to basolateral transfer. Further, the basal to apical transport

was higher and the apical to basolateral transport was lower in the ABCG2-overexpressed cells compared to empty cells (**Figure 30**). This confirms the over-expression of the ABCG2-overexpressed cell line and ensures that it can be used to measure flux.

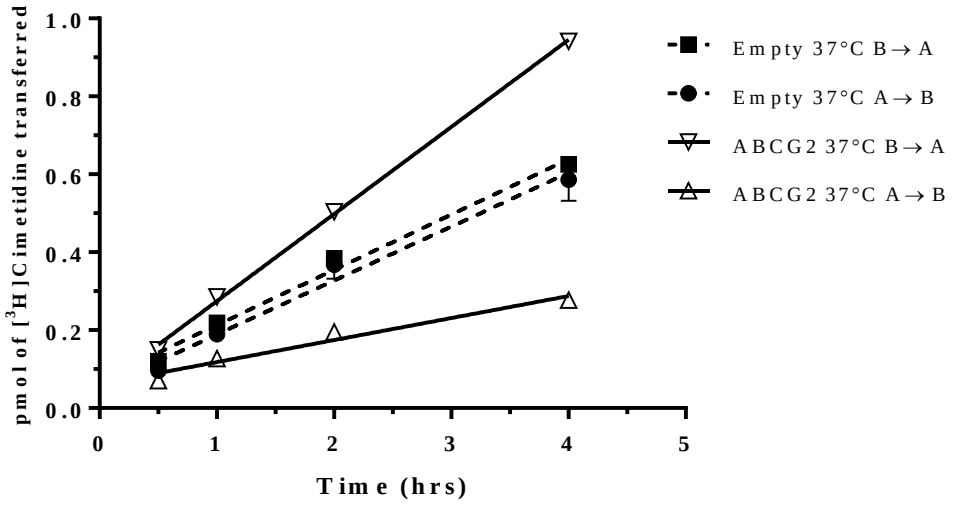


Figure 30: Flux of cimetidine across ABCG2-overexpressed and empty- MDCKII cells.

4.6.4.3 ABCC1-Overexpressed Cell Line

The basal to apical transport of [^{3}H]estradiol-17-D-glucuronide across ABCC1-overexpressed cells was significantly higher than the apical to basolateral transfer. Further, the basal to apical transport was higher and the apical to basolateral transport was lower in the ABCC1-overexpressed cells (**Figure 31**). This confirms the over-expression of the ABCC1-overexpressed cell line and ensures that it can be used to measure flux.

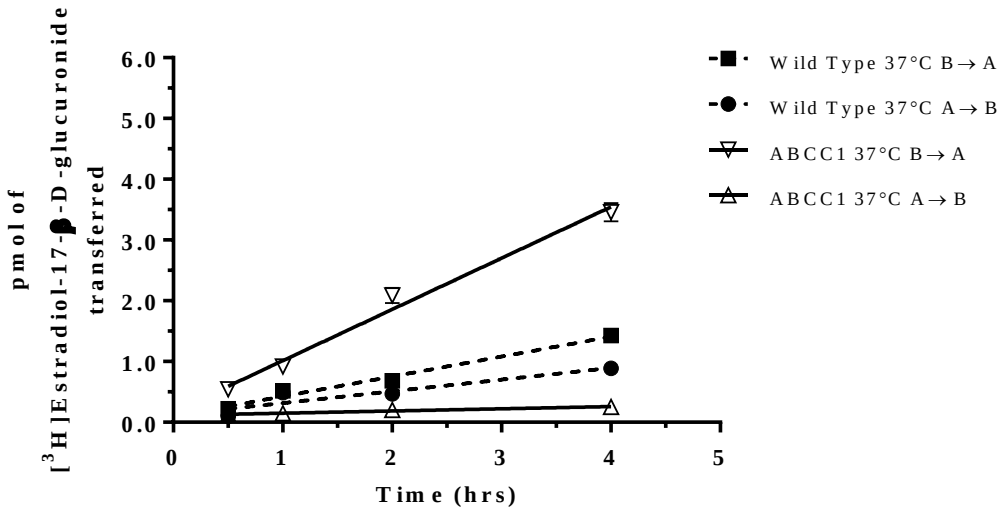


Figure 31: Flux of [^{3}H]estradiol-17-D-glucuronide across ABCC1-overexpressed and empty-MDCKII cells.

4.7 RESULTS OF PERMEABILITY FLUX ASSAYS

4.7.1 ABCB1 Flux Assays

4.7.1.1 Transepithelial Electrical Resistance

Transepithelial electrical resistance (TEER) measurements were taken on Days 1 – 4 of flux assay experiments. The TEER measurements ranged from 280 – 619 (**Figure 32**).

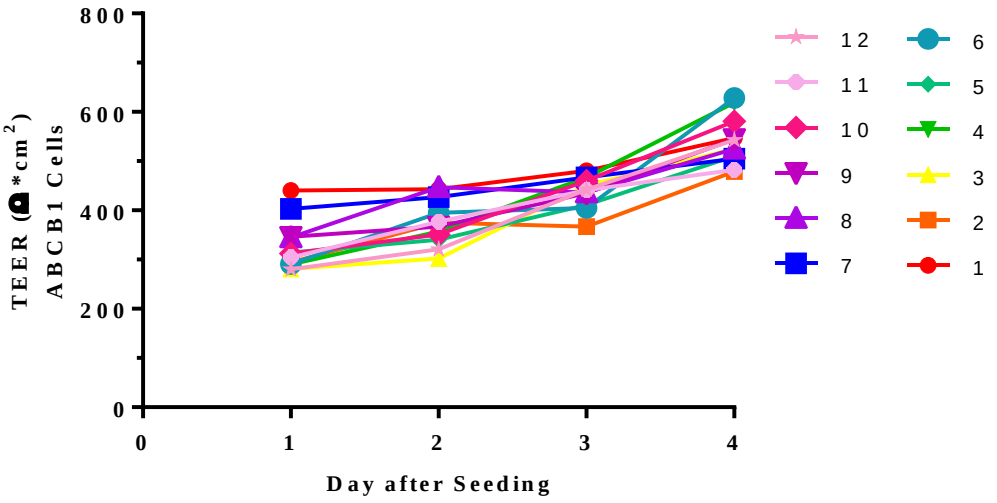


Figure 32: Transepithelial electrical measurements of ABCB1 cell monolayers.

4.7.1.2 Paracellular Transport

Paracellular transport across cell monolayers in ABCB1-overexpressed and wildtype cell lines at 40, 37, 33, and 30°C is shown in Error! Reference source not found.. A radiolabeled [^{14}C]sucrose ompound was used as a paracellular marker. Negligible [^{14}C]sucrose transport occurred across the entire four hour study duration. Less than 2% of the total [^{14}C]sucrose added crossed the cell membrane during the experiment.

4.7.1.3 ABCB1 Flux Across Cell Monolayers

The B to A transport and A to B transport of digoxin decreased with lower temperature, which indicates that ABCB1 activity was reduced at 33°C and lower (**Figure 33**).

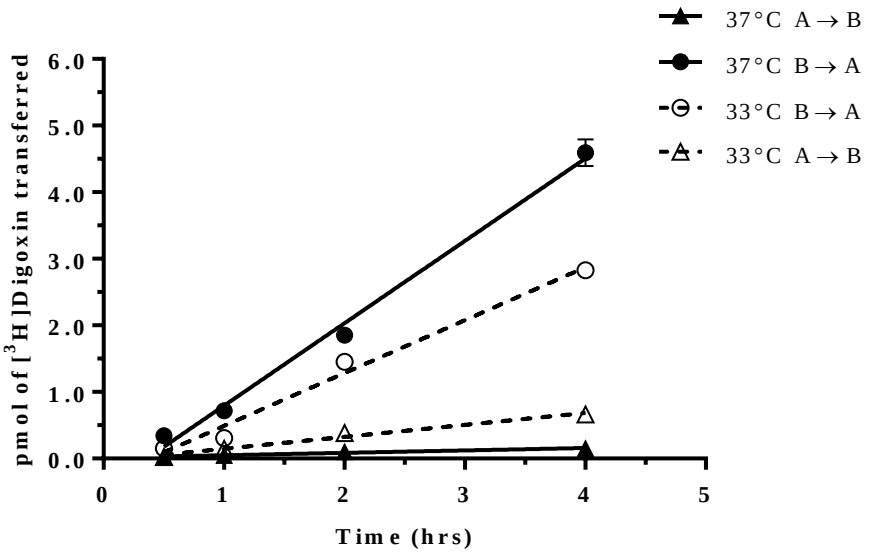


Figure 33: Digoxin flux across ABCB1-overexpressed cell monolayers at 37° versus 33°C.

4.7.2 ABCG2 Flux Assays

4.7.2.1 Transepithelial Electrical Resistance

Transepithelial electrical resistance measurements were taken on Days 1-4 of flux assay experiments. The TEER measurements ranged from 180 – 481 (**Figure 34**).

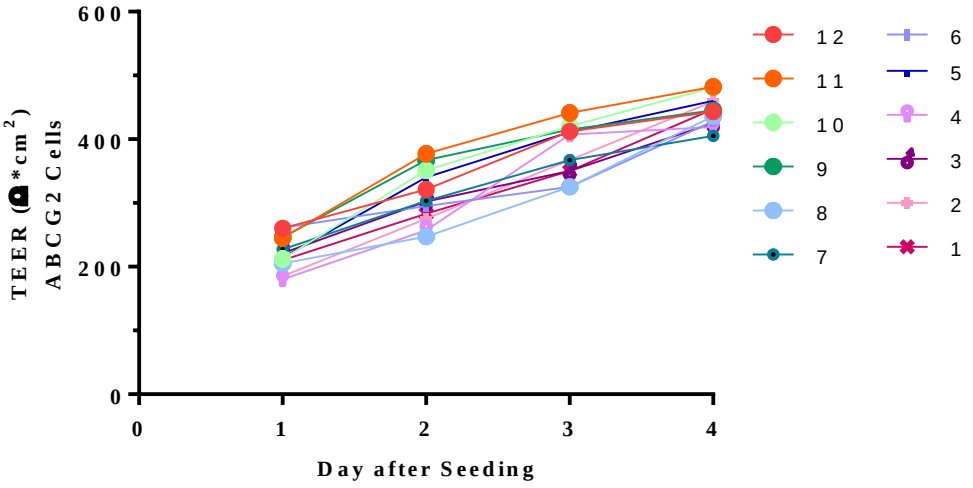


Figure 34: Transepithelial electrical resistance of ABCG2 cell monolayers.

4.7.2.2 Paracellular Transport

Paracellular transport across cell monolayers in ABCG2-overexpressed and wildtype cell lines at 40°, 37°, 33°, and 30°C is shown in **Figure 35**. A radiolabeled [^{14}C]sucrose compound was used as a paracellular marker. Negligible transport of [^{14}C]sucrose occurred across the entire four hour study duration. Less than 2% of the total [^{14}C]sucrose added crossed the cell membrane during the experiment.

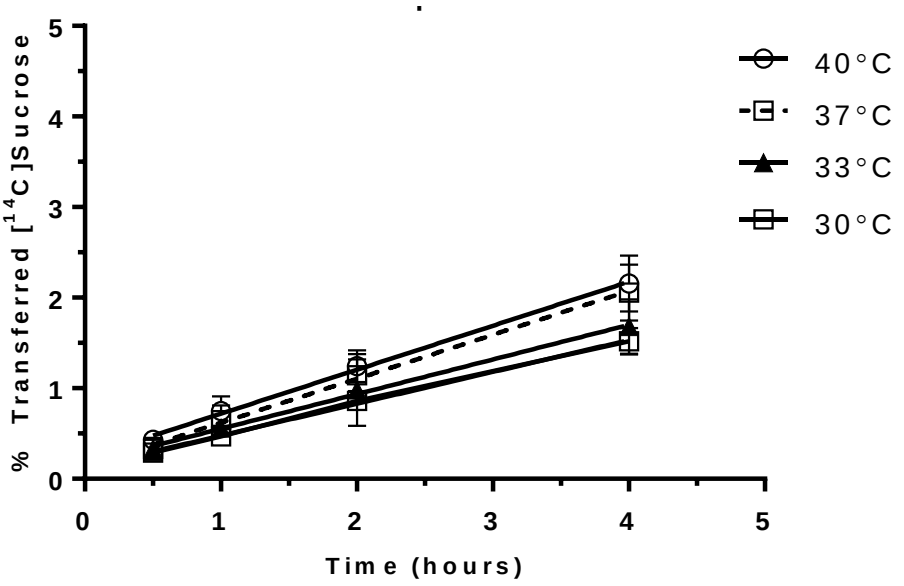


Figure 35: Transport of [^{14}C]sucrose across cell monolayers in ABCG2-overexpressed and wildtype cell lines at 40°, 37°, 33°, and 30°C.

4.7.2.3 ABCG2 Flux Across Cell Monolayers

The B to A transport and A to B transport of cimetidine decreased with lower temperature, which indicates that ABCG2 activity was reduced at 33°C and lower (**Figure 36**).

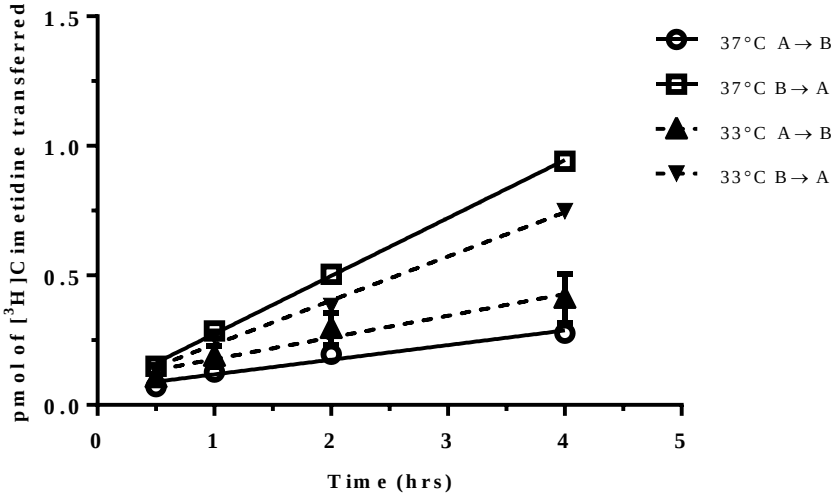


Figure 36: Cimetidine flux across ABCG1-overexpressed cell monolayers at 37° versus 33°C.

4.7.3 ABCC1 Flux Assays

4.7.3.1 Transepithelial Electrical Resistance

Transepithelial electrical resistance measurements were taken on Days 1-4 of flux assay experiments. The TEER measurements ranged from 204 – 462 (**Figure 37**).

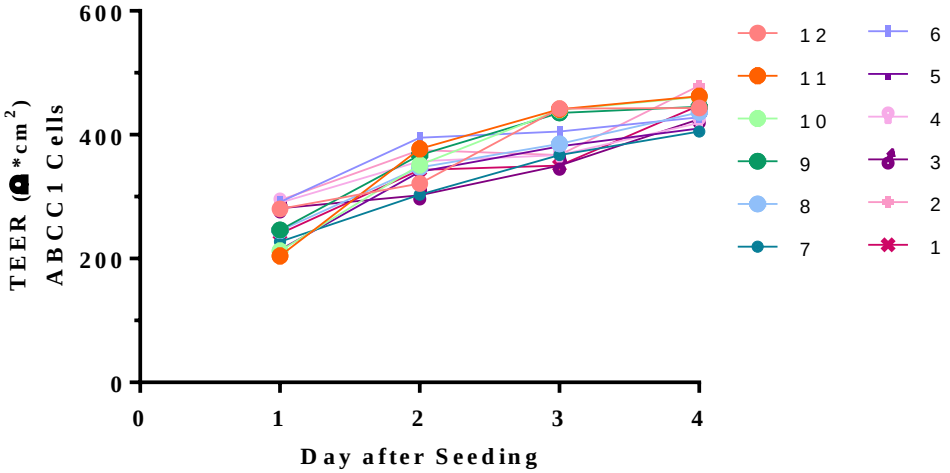


Figure 37: Transepithelial electrical resistance of ABCC1 cell monolayers.

4.7.3.2 Paracellular Transport

Paracellular transport across cell monolayers in ABCC1-overexpressed and wildtype cell lines at 40, 37, 33, and 30°C is shown in **Figure 38**. A radiolabeled [^{14}C]sucrose compound was used as a paracellular marker. Negligible transport of [^{14}C]sucrose occurred across the entire four hour study duration. Less than 2% of the total [^{14}C]sucrose added crossed the cell membrane during the experiment.

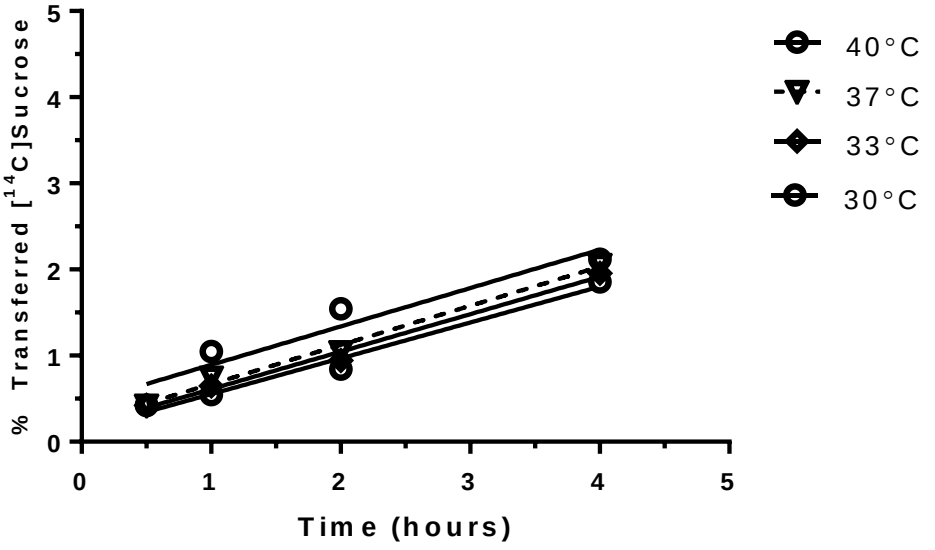


Figure 38: Transport of [^{14}C]sucrose across cell monolayers in ABCC1-overexpressed.

4.7.3.3 ABCC1 Flux Across Cell Monolayers

The B to A transport and A to B transport of [^{3}H]estradiol-17-D-glucuronide decreased with lower temperature, which indicates that ABCC1 activity was reduced at 33°C (**Figure 39**).

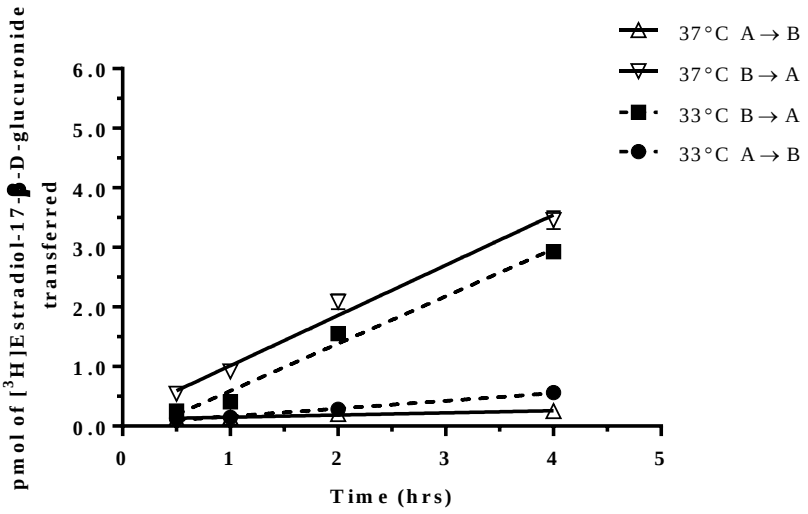


Figure 39: Estradiol-17-D-glucuronide flux across ABCC1-overexpressed cell monolayers at 37°C versus 33°C.

4.7.4 Efflux Ratios

The efflux ratio (**Equation 9**) was calculated for each over-expressed and control cell line across all temperatures. **Figure 40** demonstrates a significant decrease in the efflux ratio from 37° to 30°C in all over-expressed cell lines, which indicates a decrease in the active drug transport of these three ABC transporters. The efflux ratio decreased by 28, 32, and 26% from 37° to 33°C in ABCB1, ABCG2, and ABCC1 –overexpressed cells. No significant change in the paracellular marker, [^{14}C]sucrose, was seen in any of the cell lines or between any temperatures.

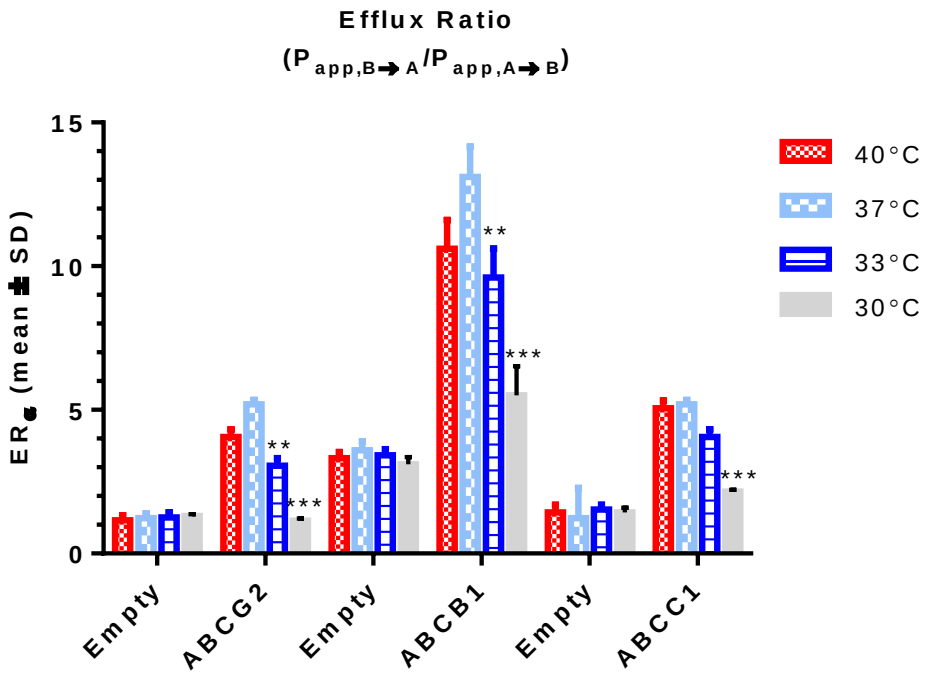


Figure 40: Efflux ratios of each probe in over-expressed and wild type cell lines at 30°, 33°, 37°, and 40°C.

Efflux ratios are measured from [^{3}H]digoxin in ABCB1-overexpressed cells, [^{3}H]cimetidine in ABCG2-overexpressed cells, and [^{3}H]estradiol-17-β-D-glucuronide in ABCC1-overexpressed cells. Two-way analysis of variance was performed (** p-value < 0.05; *** p-value < 0.01).

4.8 CONCLUSIONS

In summary, therapeutic hypothermia to 33°C decreased the active drug transport of ABCB1, ABCG2, and ABCC1, while having no significant effect on the passive transport across the cell monolayers. This decrease in active drug transport continues down to 30°C in all of the cell lines. Further, under hyperthermic conditions of 40°C active drug transport also decreased to a varying degree in each of the over-expressed ABC cell lines.

4.9 DISCUSSION

Therapeutic hypothermia has been shown to effect drug metabolism by decreasing cytochrome-P450 activity in an isoform specific manner. The aim of this study was to evaluate the effect of hypothermia and hyperthermic temperatures on the active drug transport of three ABC drug transporters (ABCB1, ABCG2, and ABCC1) at 40°C, 37°C, 33°C and 30°C. A temperature of 37°C was considered the optimal condition (normal body temperature) and significant reductions were seen in active drug transport at 33°C and 30°C. Overall, we report a decrease of 28, 32, and 26% in the active drug transport of ABCB1, ABCG2 and ABCC1, when temperature was lowered from 37°C to a mild hypothermia temperature of 33°C. No change was seen in passive transport from 37° to 33°C in any of the cell lines.

To date, there is only one *in vitro* study that has reported the effect of mild hypothermia on the active drug transport of ABCB1 (Jin, Sakaeda *et al.* 2006). In this study, Jin *et al.* investigated the effects of hypothermia on ABCB1 activity at 37°, 33°, 30°, and 4°C. The authors report a decrease in the active transport of digoxin, a specific ABCB1 probe, from 37° to 33°C (clinical target temperature), which continues to decrease down to 4°C. The authors report a decrease in active transport of 50% from 37° to 33°C, which is approximately twice as high as the decrease in transport activity we observed. One possible explanation for the difference in magnitude of transport activity is the different cell lines used in these studies. Jin *et al.* used a porcine cell line (LLC-PK1), while we used a MDCKII cell line; Literature provides evidence of differences in endogenous levels of ABCB1 between these two cell lines (Kuteykin-Teplyakov, Luna-Tortós *et al.* 2010).

In addition to hypothermia-mediated alterations on drug transport, we also investigated the effect of hyperthermia (40°C) on the active drug transport of ABCB1, ABCG2, and ABCC1. Critically ill patients are at high risk for whole-body hyperthermia, since a fever is a normal host response to infection or acute injury (Kluger 1991). During therapeutic hypothermia the subject's body temperature is actively maintained below normal body temperature, thus preventing hyperthermic conditions. However, many of the subjects present with temperatures above normal body temperature and in some cases, spikes in body temperature can be seen even throughout phases of hypothermia treatment. Thus, hyperthermia-mediated effects on drug transport activity are important to understand PK changes in these patients. Under fever conditions, the core body temperature may range from 37° to 41°C (Kregel, Wall *et al.* 1988, Erten, Saka *et al.* 2005). Thus, we chose to investigate drug transport activity at 40°C. We found a temperature increase from 37°C to 40°C led to a significant decrease in the active drug transport of ABCB1 and ABCG2 (19.1% and 21.8%), but a non-significant decrease in ABCC1 (2.5%). This implies that the optimal drug transport activity occurs at 37°C with reductions in transport activity under either hypothermia or hyperthermic conditions.

The influence of heat on drug transport activity is of considerable interest, but many of the underlying mechanisms are yet to be fully understood. While we report changes in drug transporter activity in these studies, a number of other cellular mechanisms may also be impacted by temperature, of which may contribute to overall changes in transporter-mediated drug. We discuss here literature that has investigated the effects of heat on gene expression; on the phosphorylation of drug transporter proteins; and on the permeability of epithelial

tight junctions. Many of these studies describe effects on ABCB1 (P-gp), but these changes may also be seen to a varying extent across other drug transporter proteins.

In addition to hyperthermia-mediated effects on transporter activity, the effect of hyperthermia-mediated effects on gene expression must also be taken into consideration. Hyperthermia has the potential to induce or enhance the MDR phenotype, however the effect of hyperthermia on the expression of MDR genes has not been clearly delineated. Stein *et al.* reported a strong up-regulation of P-gp and MRP1 protein levels following hyperthermia (Stein, Jurchott *et al.* 2001). Furthermore, hyperthermia led to an increase in the transcription of MDR1 and MRP1, which led to elevated levels of the corresponding proteins as well as an increase in efflux pump activity. The influence of hyperthermia on gene expression, such as MDR genes, is another factor that may contribute to overall effects of hyperthermia on drug transporter activity. The extent and degree of affect may vary depending on the length and depth of hypo- /hyper- thermia achieved. In the current study, cell lines were exposed to 4 hours under hyper- and hypo- thermic conditions to assess transport activity. While transcriptional alterations were not assessed in this study, future studies can expand on our assessment of transport activity to investigate changes in functional expression.

In addition to potential changes in gene expression, the phosphorylation of drug transporter proteins may also be modified with changes in temperature. A pre-clinical study by Yang *et al.* investigated the effect of heat shock on the activation of P-glycoprotein phosphorylation (Yang, Chin *et al.* 1995). Yang *et al.* reported that heat shock increased the phosphorylation of P-gp in MDR human breast cancer cells. They further suggest that an increase in phosphorylation of P-gp may result in an increase in its transport function. In support of this claim, several other studies demonstrated that heat shock led to an increase in

resistance of certain chemotherapeutic drugs which are substrates of P-gp in human kidney carcinoma cells and breast cancer cells (Chin, Tanaka *et al.* 1990, Ciocca, Fuqua *et al.* 1992). These results indicate that an increase in temperature may lead to an increase in transporter activity by increasing the phosphorylation of P-gp. Moreover, it's unknown how the mechanism by which the phosphorylation of P-gp is initiated. However, this interplay can further contribute to the overall effects of temperature, specifically hyperthermia, on transporter activity.

We observed no change in passive transport across any of the temperatures (30°, 33°, 37° and 40°C) and within any of the cell lines. Several studies have investigated the epithelial tight junction permeability under physiological relevant temperatures (Shapiro, Alkan *et al.* 1986, Lambert, Gisolfi *et al.* 2002, Dokladny, Moseley *et al.* 2006). Dokladny *et al.* investigated the effect of temperature changes (37° to 41°C) on intestinal epithelial tight junction permeability *in vitro* using Caco-2 intestinal epithelial monolayers (Dokladny, Moseley *et al.* 2006). Results showed that an increase in temperature from 37° to 41°C led to a significant disturbance in intestinal epithelial tight junction barrier. Further, results demonstrate that expression of heat-shock protein plays an important protective role in preventing disruption of the intestinal TJ barrier when under heat-induced stress. Another *in vitro* study investigated the effect of temperature (heat only) on epithelial permeability, using a radiolabeled mannitol probe (paracellular marker), in MDCK cells (Moseley, Gapen *et al.* 1994). At temperatures above 37°C, a temperature-dependent increase in epithelial permeability was observed. Furthermore, hyperthermia leads to a number of cellular effects which may contribute to altering epithelial permeability such as changes in membrane

fluidity, phospholipase C (PLC) activation, and alterations in cytoskeletal microfilaments (Laszlo 1992).

In addition to the affects described above, hyperthermia may lead to a number of other physiologic and mechanistic effects, similarly to what was described for hypothermia in Chapter 1. Whole body hyperthermia causes a reduction in blood flow to the GI tract (Kregel, Wall *et al.* 1988), which allows for a greater cardiac output to reach the periphery for heat dissipation (Rowell 1974). Additional hyperthermia-mediated affects include hypoxia (Hall, Baumgardner *et al.* 1999), free radical production (Hall, Buettner *et al.* 1994, Hall, Buettner *et al.* 2001), ATP depletion, and cellular dysfunction (Gisolfi 2000). Collectively, these results demonstrate the intricate interplay of temperature-mediated affects at the cellular level, which may contribute to different affects seen *in vivo* than *in vitro*.

There are several limitations to this study. First, this is an *in vitro* analysis to investigate changes in drug transport, and while it allows for absolute quantification of each independent drug transporter, the magnitude of hypothermia-mediated decreases in drug transport may not directly correspond to clinical changes. Additionally, these results describe the magnitude of change of each independent transporter. However, clinical changes will constitute a variety of other covariate effects that may affect drug transport activity. Many of the temperature effects both mechanistically and physiologically will contribute to varying extents *in vivo*. Further, injury is also known to affect drug transport activity and may further affect magnitude of change seen in patients.

In addition to temperature-mediated effects on active drug transport, there is also a potential for injury-mediated affects. Zhou *et al.* investigated the effect of therapeutic hypothermia and disease on CYP450 pathways in a rat model of cardiac arrest. In this pre-

clinical study, cardiac arrest was shown to have an effect on drug metabolism via CYP450 pathways as demonstrated by several different probe drugs (Zhou, Empey *et al.* 2011). Future analysis is warranted to determine if these injury related affects carry over to drug transporter pathways as well.

Injury-mediated effects on drug transporter activity have also been described following brain injury, such as ischemic, hemorrhagic, or traumatic (Chodobski, Zink *et al.* 2011). Following brain injury, changes in blood-brain barrier permeability may occur, in addition to other pathophysiological factors, which in combination may be attributed to an overall post-traumatic dysfunction of the blood-brain barrier. The activity of efflux transporters at the BBB is one important factor which controls whether neuroprotective drugs can reach the brain (Loscher and Potschka 2005). Many of the molecular mechanisms that contribute to the regulation transporter activity are not fully understood, however some factors have been identified, such as proinflammatory mediators and ROS, which play a role in this regulation (Loscher and Potschka 2005, Potschka 2010). In a mouse model of cerebral ischemia, ischemic brain injury was associated with an upregulation of ABCB1 expression (Spudich, Kilic *et al.* 2006). While evidence on blood-brain barrier disruption following cardiac arrest is more limited, pre-clinical evidence demonstrates that under hypoxic-ischemic conditions disruption of the BBB occurs (Kaur and Ling 2008). Moreover, injury-mediated changes are expected in addition to temperature-mediated changes. The combination of disease and hypothermia may lead to different effects on drug transport. Future studies should investigate how injury-mediated versus hypothermia-mediated effects play a role on drug transport. Additionally, a focus on post-cardiac arrest effects on drug transport at the blood brain barrier is a future area of study.

In addition to ABCB1, ABCG2, and ABCC1, various other drug transporters, both of the ABC and SLC superfamilies, can play a role in drug disposition *in vivo*. Further studies should investigate the combination of other drug transporters. In particular, other transporters that are known to have emerging clinical important such as ABCC2, ABCC3, and ABCC4 would be important to evaluate (Giacomini, Huang *et al.* 2010). In addition, the interplay of therapeutic hypothermia and injury-mediated effects on drug pharmacokinetics, both from a metabolic and transporter-mediated pathway, should be further investigated to understand how each plays a role in overall drug disposition. Overall, we report a decrease in the active drug transport of ABCB1, ABCG2, and ABCC1 from 37°C to 33°C and 30°C. Determining the translation of theses affects from an *in vitro* to an *in vivo* system will be an important next step.

4.10 CLINICAL RELEVANCE

While therapeutic hypothermia has been shown to effect drug metabolism, the effects on other components of drug pharmacokinetics such as drug transporters are still largely unknown. Drug transporters play a significant role in drug disposition. The International Transporter Consortium has provided recommendations for which drug transporters are clinically important and which methods are best for studying these drug interactions (Giacomini, Huang *et al.* 2010, Hillgren, Keppler *et al.* 2013). The following sections discuss the clinical relevance of each of the three ABC drug transporters (ABCB1, ABCG2, and ABCC1) discussed in this Chapter.

4.10.1 ABCB1

ABCB1 (more commonly known as P-glycoprotein or P-gp) is an ATP-dependent drug transporter within the ABC superfamily of drug transporters. ABCB1 is expressed in a number of tissues including the luminal membrane of the small intestine and blood-brain barrier; the apical membranes of excretory cells such as hepatocytes; and kidney proximal tubule epithelia. ABCB1is known to play an important role in regulating the entry of xenobiotics into the central nervous system (Choudhuri and Klaassen 2006, Raub 2006, Chinn and Kroetz 2007, Kimura, Morita *et al.* 2007, Miller, Bauer *et al.* 2008, Zhou 2008, Giacomini, Huang *et al.* 2010).

The International Transporter Consortium lists the following P-gp-mediated drug-drug interactions: quinidine-digoxin; ritonavir-digoxin; dronedarone-digoxin; and ranolazine-digoxin (Giacomini, Huang *et al.* 2010). Currently the majority of the data surrounding P-gp-mediated drug-drug interactions is pre-clinical. Clinical evidence is limited for these drug-interactions and inconsistent as to whether inhibition of P-gp at the blood brain barrier leads to adverse events. Furthermore, studies on ABCB1 polymorphisms on P-gp substrates also demonstrate conflicting results, which makes it unclear if polymorphisms in ABCB1 may also contribute to drug-drug interactions.

4.10.2 ABCG2

ABCG2 (more commonly known as the breast cancer resistance protein, or BCRP) is considered a half ABC drug transporter (Wakabayashi, Tamura *et al.* 2006, Giacomini, Huang *et al.* 2010). ABCG2 is expressed in many tissues within the body including the gastrointestinal tract, liver, kidney, brain endothelium, mammary tissue, testis and placenta. Like ABCB1, ABCG2 plays a

role in regulating transport across the blood-brain barrier, as well as transport across the blood-testis barrier and the maternal-fetal barrier (van Herwaarden and Schinkel 2006, Vlaming, Lagas *et al.* 2009). Additionally, ABCG2 plays a role in absorption by limiting oral bioavailability.

The International Transporter Consortium suggests a potential ABCG2-mediated drug-drug interaction between GF120918 and Topotecan (Giacomini, Huang *et al.* 2010). Additionally, clinical studies have demonstrated that a decrease in ABCG2 activity may lead to altered pharmacokinetics of diflomotecan, irinotecan, rosuvastatin, sulphasalazine, 9-aminocamptothecin and topotecan (Cusatis, Gregorc *et al.* 2006, Zhang, Yu *et al.* 2006, Cusatis and Sparreboom 2008, Polgar, Robey *et al.* 2008, Yamasaki, Ieiri *et al.* 2008, Keskitalo, Zolk *et al.* 2009). While evidence is still very limited, changes in BCRP activity and function could potentially lead to changes in drug absorption, exposure and distribution for drugs that are substrates of ABCG2.

4.10.3 ABCC1

ABCC1 (more commonly known as the multidrug resistance protein 1 or MRP1) is a full ABC drug transporter located ubiquitously in tissues with highest expression in testis, lung, kidney, cardiomyocytes, placenta, prostate and lower expressions in small intestine, colon, and brain (Rosenberg, Mao *et al.* 2001, Zhang, Schuetz *et al.* 2004, Bakos and Homolya 2007). ABCC1 is known to play a role in multi-drug resistance in various types of cancer. Currently there are no significant evidence of a role in drug metabolism or absorption; however it may play a role in chemotherapeutic agents and drug distribution. It has been shown to affect drug distribution in tissue and it is believed to play a role in the lack of response from chemotherapeutic drugs (Wijnholds, Evers *et al.* 1997, Mueller, Widder *et al.* 2005).

5.0 CONCLUSIONS AND FUTURE DIRECTIONS

5.1 CONCLUSIONS

5.1.1 Key Research Findings

The purpose of this research was to investigate the effects of therapeutic hypothermia on drug metabolism and ABC-drug transport. In order to assess drug metabolism, an analytical method was first developed to quantify phenytoin and levetiracetam concentrations in serum. Furthermore, this method was applied to a clinical study to quantify serum samples for pharmacokinetic analysis. Additionally, a population pharmacokinetic analysis was developed to evaluate the effect of therapeutic hypothermia on phenytoin elimination in pediatrics following cardiac arrest. In order to begin to systematically investigate the effects of therapeutic hypothermia on drug transport, an *in vitro* cell culture model was utilized to examine these effects on ABCB1, ABCG2, and ABCC1. Specifically, MDCKII cells were utilized to examine the effect of hypo- and hyper- thermia (30, 33, 37, 40°C) on active and passive transport across cell monolayers.

Results from Chapter 2 demonstrate that a sensitive and specific method was successfully developed to quantify low volume serum samples. This method is the first to report quantification of both phenytoin and levetiracetam in serum volumes as low as 20 µL. From this

analytical method, we accurately quantified serum samples from pediatrics (n = 36) receiving anti-epileptic drug and simultaneously undergoing therapeutic hypothermia.

Chapter 3 extends the method developed in Chapter 2 by investing the effect of therapeutic hypothermia on the metabolism of phenytoin in pediatrics following cardiac arrest. This chapter describes the population pharmacokinetic model that was developed to best describe phenytoin levels in this patient population consisting of pediatrics after cardiac arrest. A 1-compartment Michaelis-Menten model best described phenytoin concentration in this population, which is consistent with previous models of phenytoin pharmacokinetics. Following covariate model building, weight and temperature were found to be significant on the maximum velocity of phenytoin. Visual predictive checks confirmed that the predicted model concentrations well described the observed data.

In order to expand what's known about hypothermia-mediated effects on drug disposition, we performed permeability flux assays in MDCKII-cell line overexpressing specific drug transporters. Chapter 4 describes the development of assay and experimental conditions to investigate how temperature (clinically applicable hyperthermic and hypothermic conditions) effect drug transporters in a cell culture model. Interestingly, both hypothermic and hyperthermic conditions decreased overall active transport activity in all three drug transporters, ABCB1. ABCG2, and ABCC1, but did not significantly change the passive transport across the cell membranes. Collectively, these findings demonstrate that in addition to drug metabolism, therapeutic hypothermia decreases active drug transport activity *in vitro* and may contribute to changes in drug disposition *in vivo*.

5.2 FUTURE DIRECTIONS

5.2.1 Future Studies and Potential Areas for Discovery

The implementation of therapeutic hypothermia has continued to evolve over the past decade as we have learned more about how to safely and effectively administer this intervention. As a result, new recommendations for the application of therapeutic hypothermia have been advised as we learn more about the benefits and risks of cooling patients and the expanding sophistication of PK evaluations in critical care. In the initial implementation of therapeutic hypothermia, the depth of cooling varied considerably across pre-clinical and clinical studies (28 – 34°C). Many initial clinical and pre-clinical studies cooled to temperatures well below the current optimal target clinical range, to concentrations more likely to be associated with adverse events. In addition, the majority of the patient population was brain-injured patients or healthy volunteers, instead of the patient populations recommended for therapeutic hypothermia, CA patients and neonates with HIE. Further, over the past ten years the need for more robust population PK analysis approaches (pharmacometrics) has been recognized and applied (Poloyac and Empey 2013). Initial studies reported mostly surveys of drug concentrations. However, more recent studies have conducted true PK evaluations and a number of clinical studies have used more robust pharmacometric approaches to investigate the effect of temperature versus other covariates on PK parameters.

Recent studies suggest that the magnitude of change during hypothermia is impacted by the fact that ischemic insults, or changes in physiology such as blood flow, also reduce drug metabolism. As outlined in Chapter 1, therapeutic hypothermia decreases Phase I drug metabolism of the CYP450 enzymatic pathways leading to an increase in the blood

concentrations of many of these drugs. However, the change in drug PK may be attributed to a combined effect of injury and hypothermia. Previously our laboratory demonstrated that chloroxazone and midazolam clearance decreased in a hypothermic rat model of CA as compared to sham normothermic rats (Zhou, Empey *et al.* 2011). Population pharmacokinetic modeling demonstrated that an interaction between hypothermia and cardiac arrest led to an even greater effect on metabolism than in the presence of either covariate alone. In addition many clinical studies, such as Filippi *et al.*, speculated that asphyxia was contributing to PK changes in addition to hypothermia. Some studies, such as van den Broek *et al.*, found no effect of temperature on drug PK but did find changes in clearance parameters in asphyxia neonates when compared to non-asphyxia neonates in the literature.

Based on recent evidence evaluating rewarming and post-treatment phases, the theoretical time course of hypothermia-mediated effects on drug disposition should be revised. We previously described a decrease in CYP450 activity during cooling followed by a return to basal activity following the rewarming phase. However, newer data clearly demonstrate that consequences of metabolic effects may persist even after the rewarming phase and into the post-treatment period. This was seen in the study by Empey *et al.* demonstrating prolonged effects of reduced phenytoin elimination well into the post-treatment period. Additionally Bjelland *et al.* saw no change in fentanyl concentration during the rewarming phase and attributed it to a possible delay in metabolic changes due to its long half-life. Shellhaas *et al.* also speculated that PB's long half-life may attribute to changes even after rewarming. Heightened awareness and increased monitoring of hypothermia effects should thus be taken into consideration even after cooling is stopped and during the rewarming and post-treatment phases, particularly for drugs with long half-lives.

In conclusion, recent data confirm that hypothermia, such as that employed during therapeutic hypothermia, alters PK and elevates drug concentrations for several medications commonly used in the intensive care unit. In contrast to early studies, recent clinical investigations have 1) incorporated robust PK methods such as pharmacometrics and 2) conducted experiments using recommended clinical protocols (degree of cooling and duration of cooling/rewarming) and 3) incorporated appropriate comparator/control groups. The current data demonstrates a combined effect of injury and hypothermia on drug PK and response. Clinical studies are on-going to evaluate specific dosing regimens in critically ill patients undergoing therapeutic hypothermia across various levels of hypothermia (clinicaltrials.gov; NCT01560338, NCT02546947, NCT02529202, NCT02621944, NCT02252848). The effects of hypothermia and injury on drug disposition continue to be an important consideration when dosing and monitoring patients undergoing targeted therapeutic hypothermia. Finally, additional well-designed studies evaluating the impact of hypothermia on drug transport and pharmacodynamics are needed.

5.2.2 Targeted Temperature Management versus Therapeutic Hypothermia

In the largest randomized control trial of TTM to date, Nielson *et al.* reported that cooling to 36 versus 33°C does not have a significant difference on the outcome of out-of-hospital CA patients (Nielsen, Wetterslev *et al.* 2013). These results suggest that the degree to which adult CA patients are cooled may lessen, which could potentially diminish the effects of cooling on drug disposition. However, as seen in the findings and interpretation of the recent pediatric out-of-hospital CA study, currently, many centers continue to cool adult and pediatric CA patients to 33-34°C and therapeutic hypothermia remains standard of care in term neonatal HIE patients.

5.2.3 Expected Trends and Research Focused on Therapeutic Hypothermia

The initial effects on hepatic drug elimination approximated an 11% decrease in systemic clearance for 1°C change in body temperature. Recent clinical and preclinical studies have verified these findings in mild hypothermia, although earlier investigations had suggested a much greater change. A discrepancy in the results of hypothermia-mediated effects can largely be attributed to changes in the hypothermia protocols. Many initial estimates were based on studies that cooled patients well below the clinically optimal temperature range and for much longer or shorter cooling periods. As guidelines have evolved to reflect the optimal benefit-to-risk ratio for active cooling, pharmacometric analysis has become more robust, using a population pharmacokinetic approach to evaluate the impact of temperature versus other potential covariates (e.g., disease severity) contributing to changes in drug disposition.

Future studies should investigate the extent of which injury versus cooling effect drug PK in clinical studies as well as the interplay of these effects on drug pharmacodynamics. Another important observation in these studies is the effect of critical care injuries on metabolism. Studies have demonstrated that critical care injuries lead to alterations in drug metabolism and disposition. Preclinical work in our lab has further demonstrated a combined effect of injury and hypothermia on drug pharmacokinetics and, in some cases, injury may be the predominant driver in pharmacokinetic changes. Evidence of a combined injury and temperature effect on drug pharmacokinetics can be seen in a number of clinical studies where pharmacometric analysis failed to demonstrate a temperature-mediated change on drug clearance, but a decrease in clearance was still seen when results were compared to those in uninjured subjects. For example, pharmacometric analysis could not identify any (clinically relevant) effect of moderate

therapeutic hypothermia on the pharmacokinetics of phenobarbital in asphyxiated neonates; however, a decrease in clearance was observed when results were compared to literature values of non-asphyxiated neonates. Most hypothermia studies demonstrate a combined effect of injury and cooling on drug pharmacokinetics; therefore, future studies should focus on determining which factor is predominately driving changes in pharmacokinetics.

Furthermore, the implementation of therapeutic hypothermia in various other patient populations should be a future area of research. To date, therapeutic hypothermia has been studied in a number of patient populations. However, it has only been shown to be efficacious in two main patient populations: adult CA patients and neonates with HIE. One reason for the lack of beneficial effects in various other populations may be due to adverse events that occur during treatment. As implementation of therapeutic hypothermia continues to evolve it is important that we continue to evaluate hypothermia treatment in various populations

Current data suggest that both liver ischemia and targeted temperature management decrease hepatic metabolism in a cytochrome P-450 isoform-specific/drug-specific manner. The magnitude of the change in metabolism will likely be most important for drugs with long half-lives and narrow therapeutic windows. In particular, the prolonged duration of action of sedatives and hypnotics should be considered when evaluating neurological function after insult. Furthermore, specific drugs that require enzymatic activation (such as clopidogrel) and other pharmacokinetic processes (such as drug transport) are important areas for study.

5.2.4 Clinical and Translational Implications

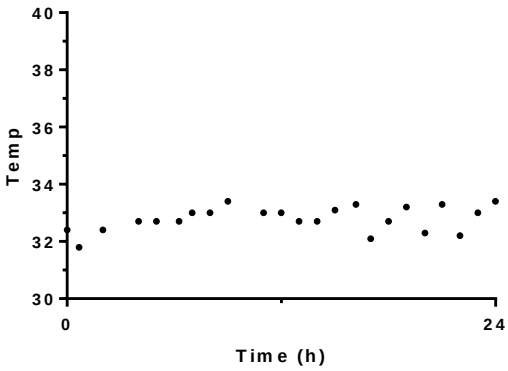
The results of the work performed throughout this dissertation could have important clinical implications. Reductions in drug metabolism have been shown to lead to super-therapeutic drug

levels, which ultimately could lead to drug toxicity and adverse events in patients treated with therapeutic hypothermia. Several recent clinical studies have begun to provide dosing recommendations during hypothermia therapy, based on the results of pharmacokinetic and/or pharmacometric analyses. In order to continue to provide dosing recommendations, more research is warranted in order to optimize patient care.

APPENDIX A: EXPERIMENTAL DATA FROM THERAPEUTIC HYPOTHERMIA IN PEDIATRIC CARDIAC ARREST STUDY

A.1. TEMPERATURE PROFILES OF INDIVIDUAL PEDIATRIC PATIENTS

The following figures depict the temperature profiles (degrees Celsius) of each pediatric patient enrolled in the study and receiving phenytoin (n = 35). Therapeutic hypothermia was administered at cooling durations of 24, 48, or 72 hours. Each dot represents an individual temperature measurement. In some cases, the patient died before the end of therapeutic hypothermia treatment.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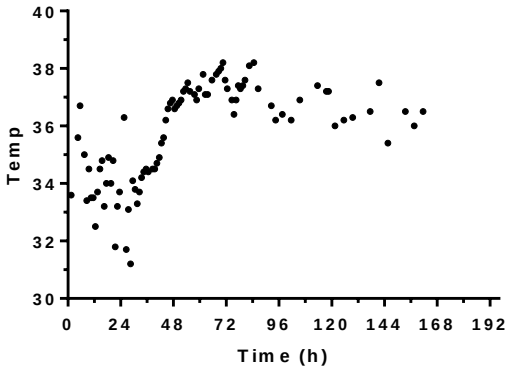
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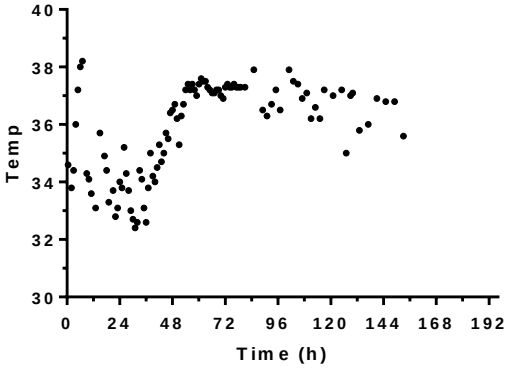
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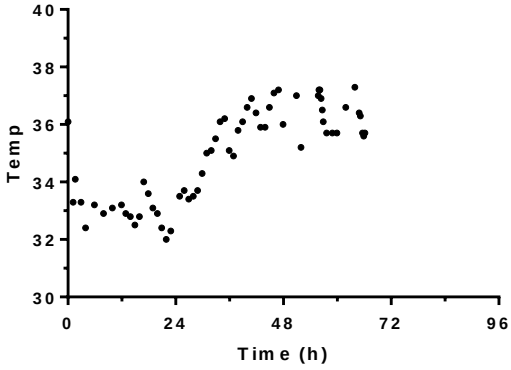
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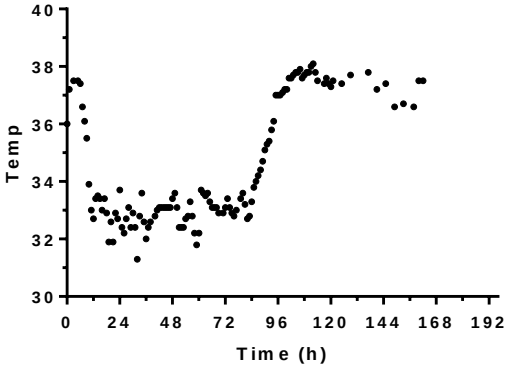
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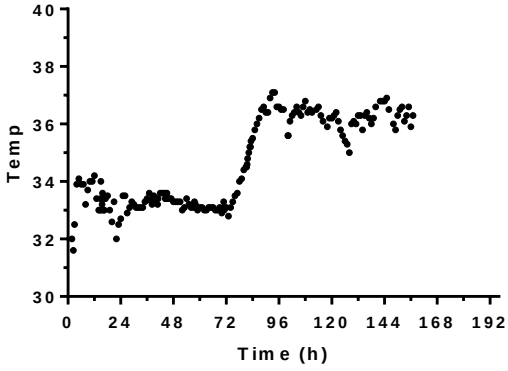
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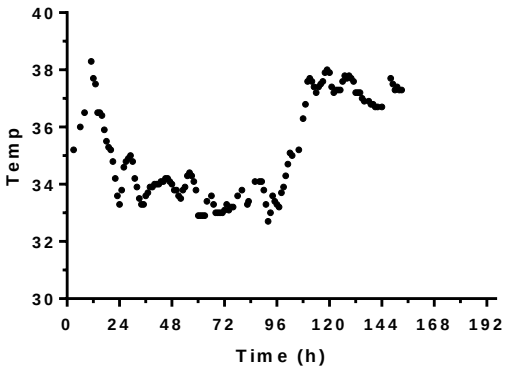
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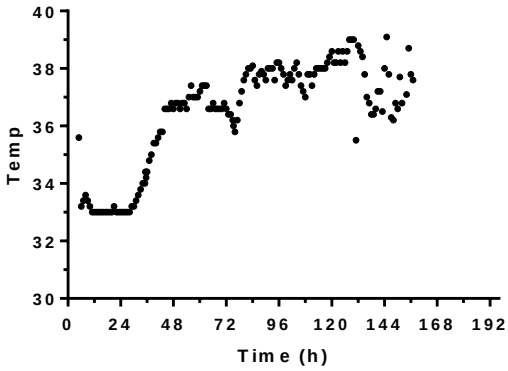
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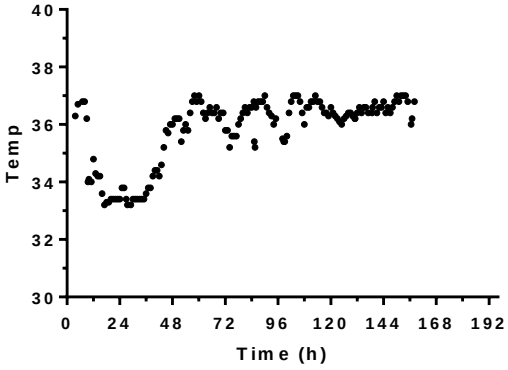
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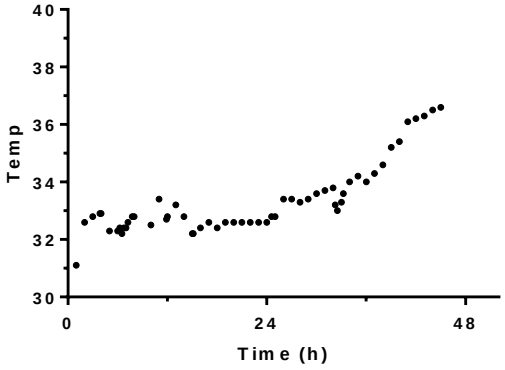
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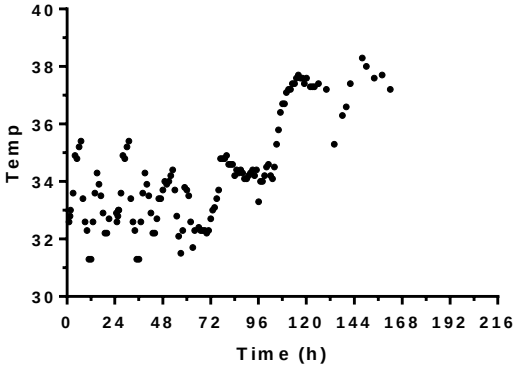
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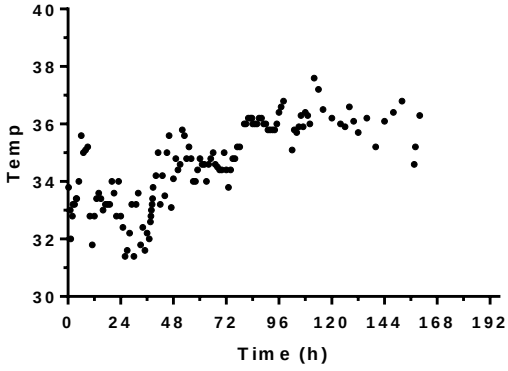
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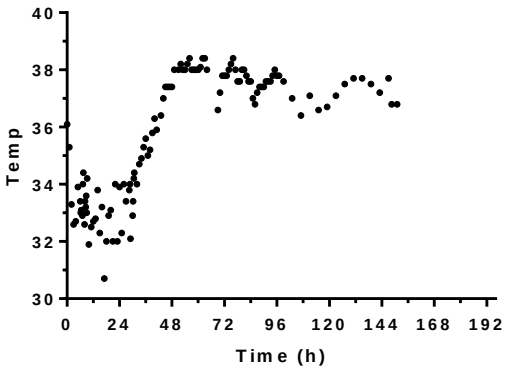
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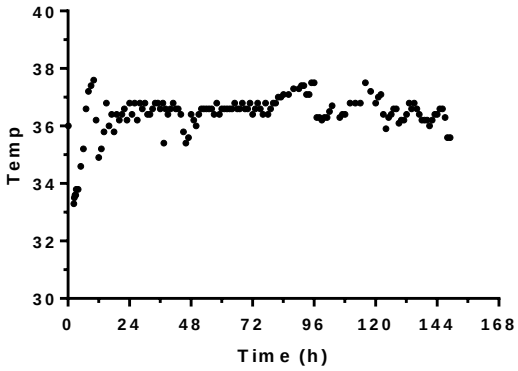
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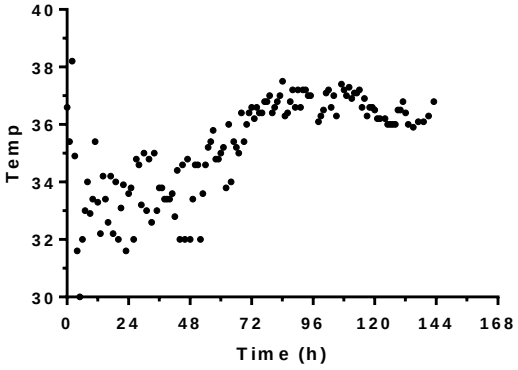
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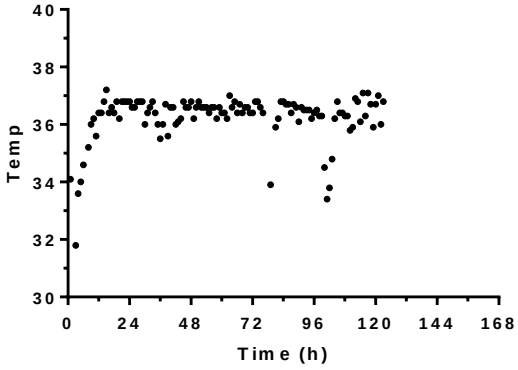
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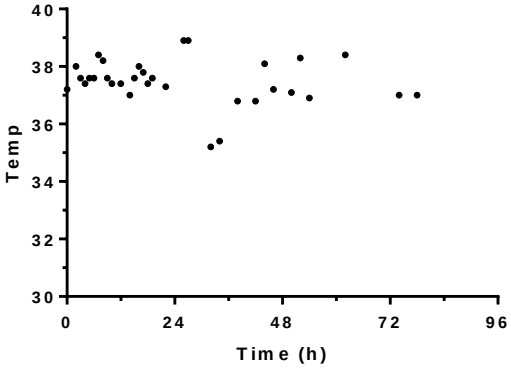
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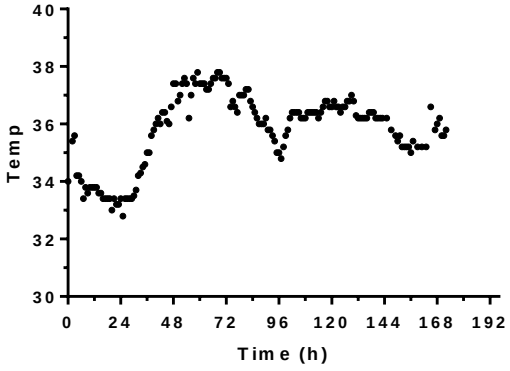
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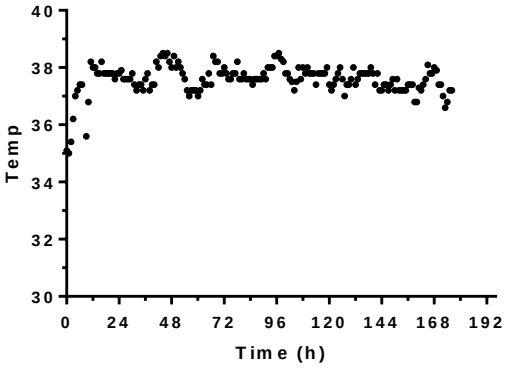
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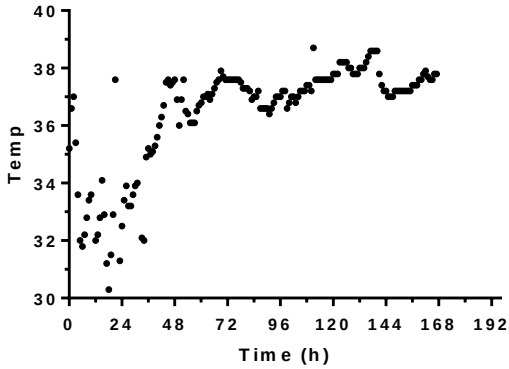
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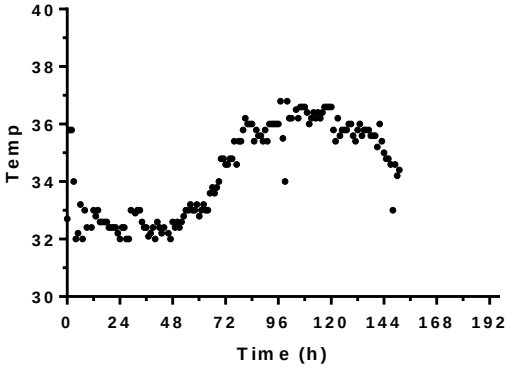
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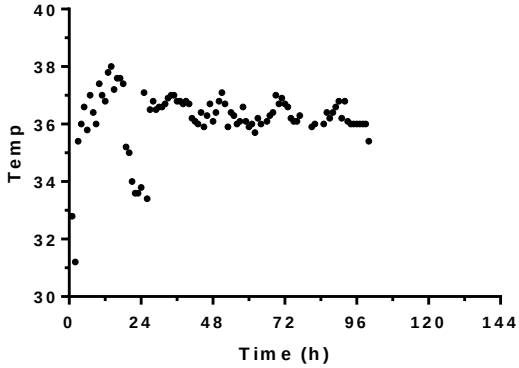
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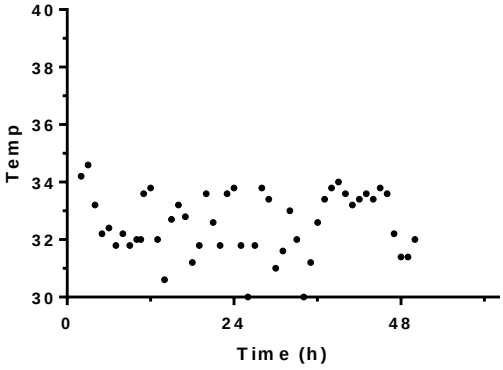
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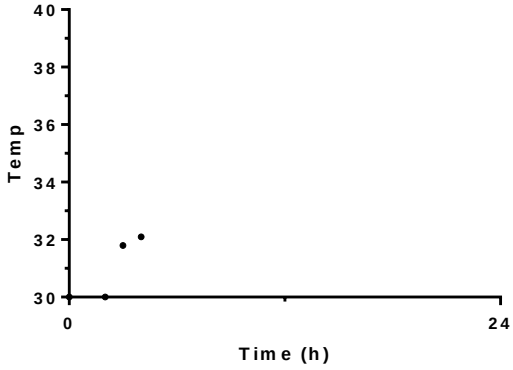
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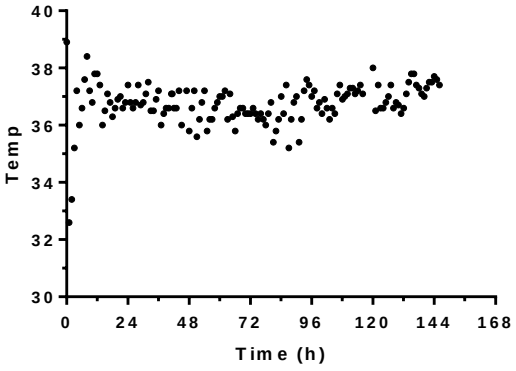
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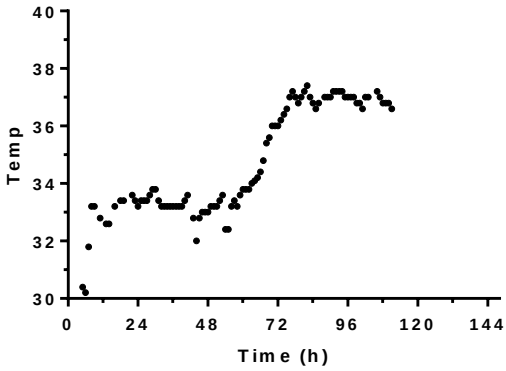
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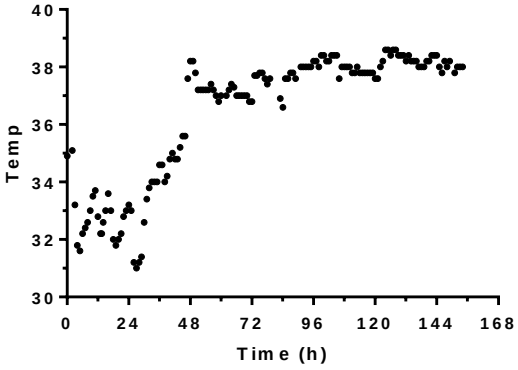
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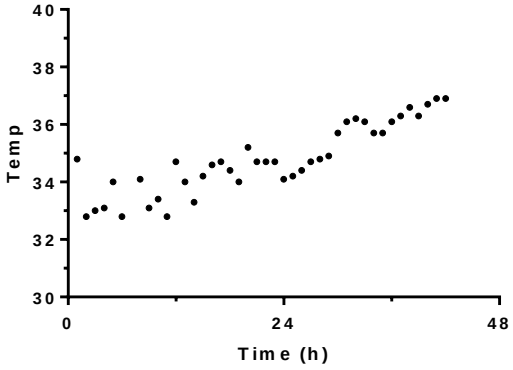
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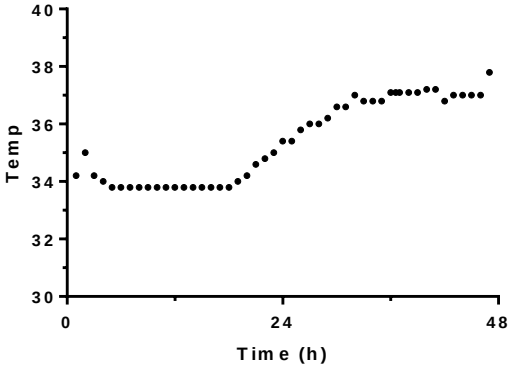
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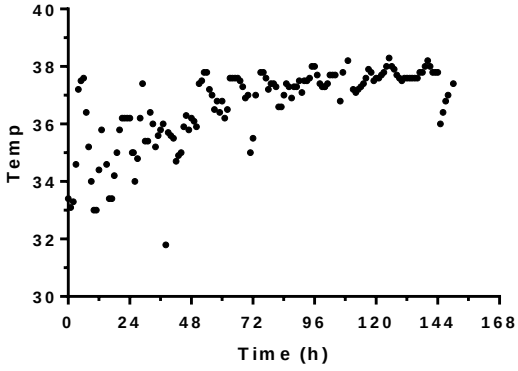
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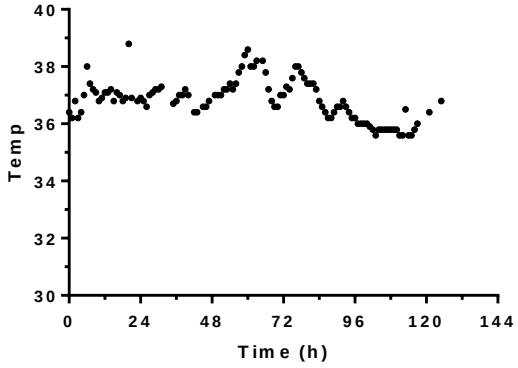
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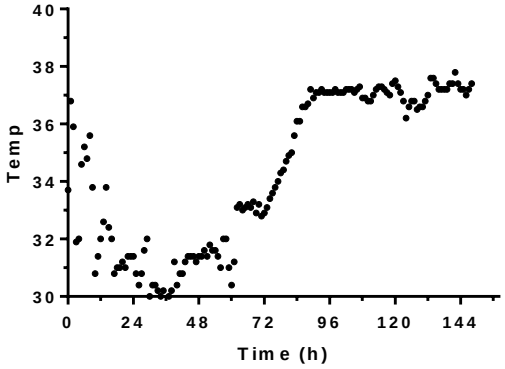
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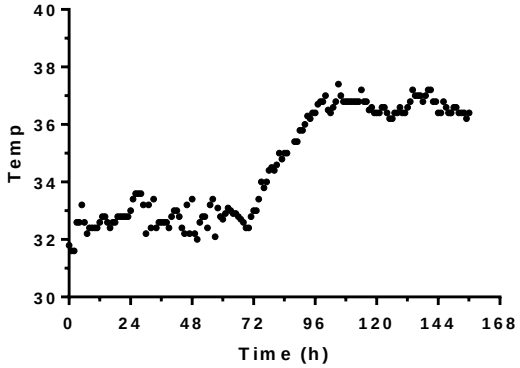
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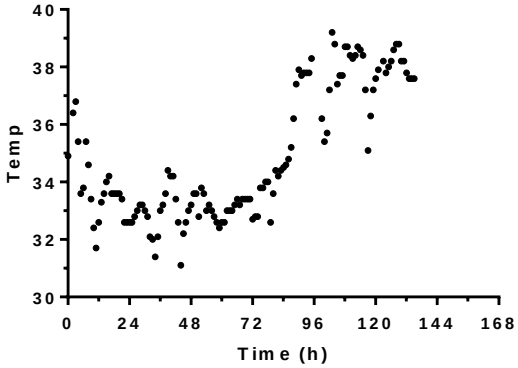
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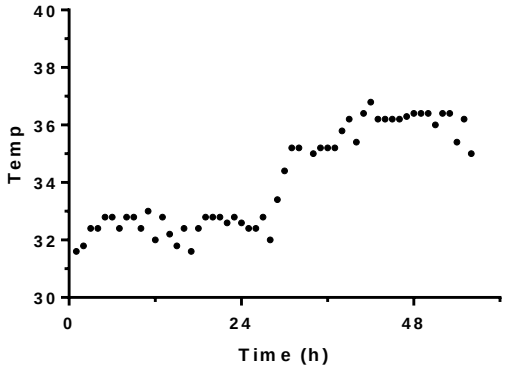
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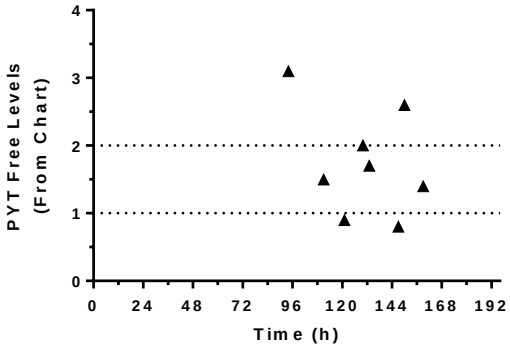
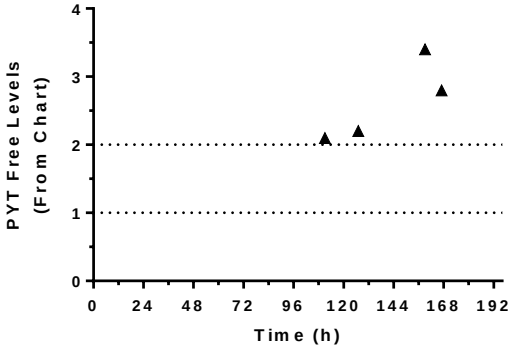
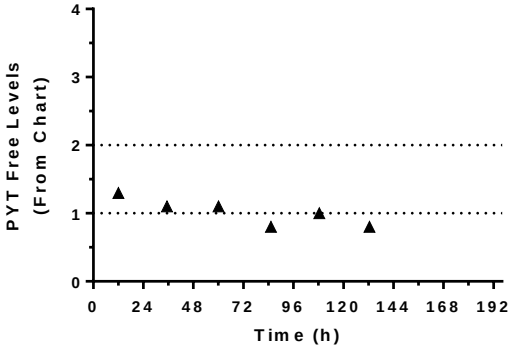
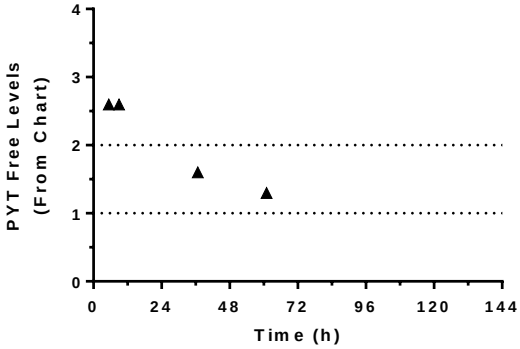
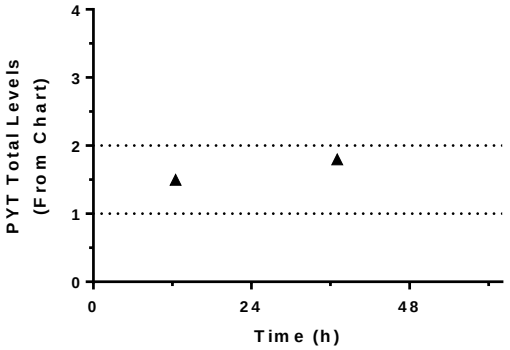
A.2. FREE PHENYTOIN CONCENTRATIONS IN INDIVIDUAL PATIENTS

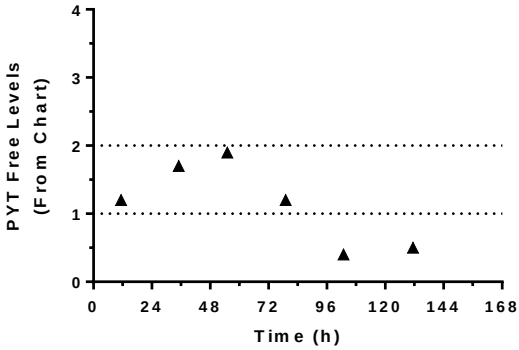


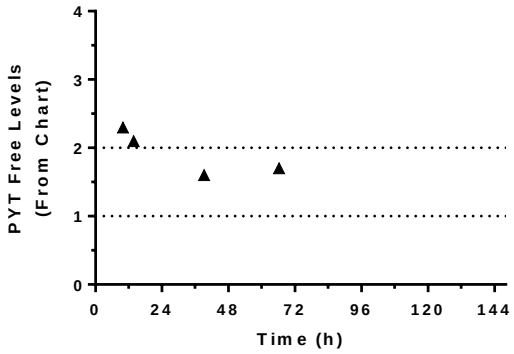








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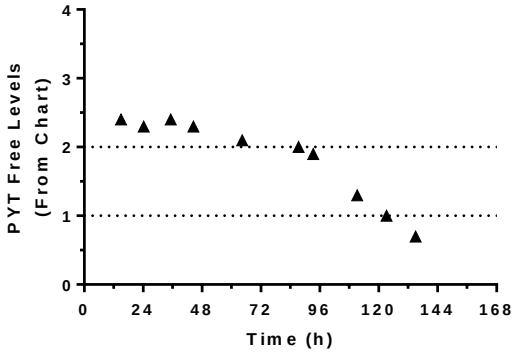
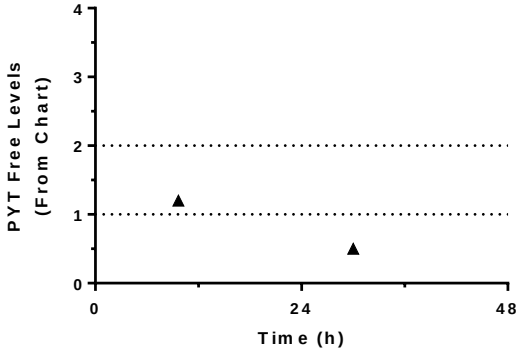
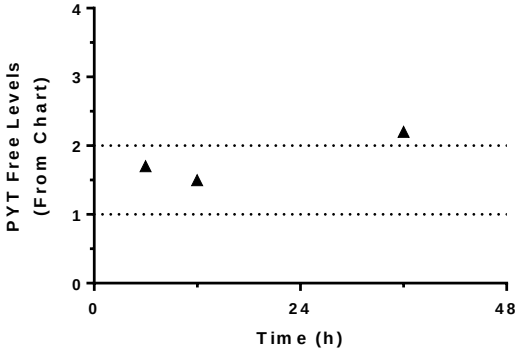
Time (h)


PYT Free Levels
(From Chart)
0
1
2
3
4
0
24
48
72
96
120
144
168
Time (h)


PYT Free Levels
(From Chart)
0
1
2
3
4
0
24
48
Time (h)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(From Chart)
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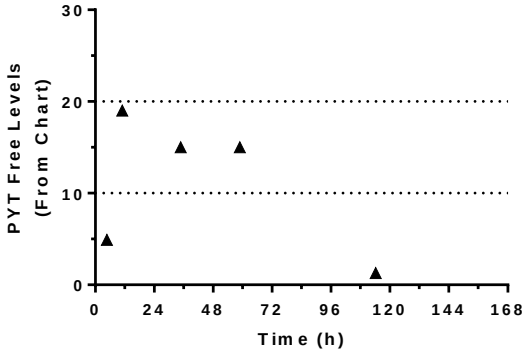
2
3
4
0
24
48
Time (h)


PYT Free Levels
(From Chart)
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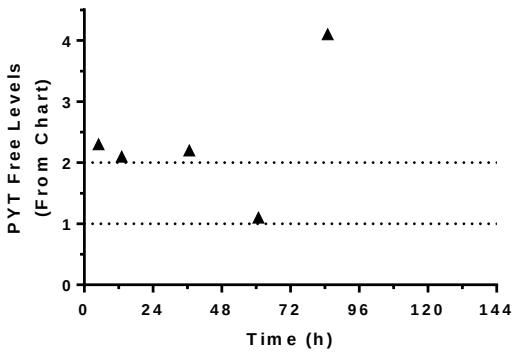
20
30
0
24
48
72
96
120
144
168
Time (h)


PYT Free Levels
(From Chart)
0
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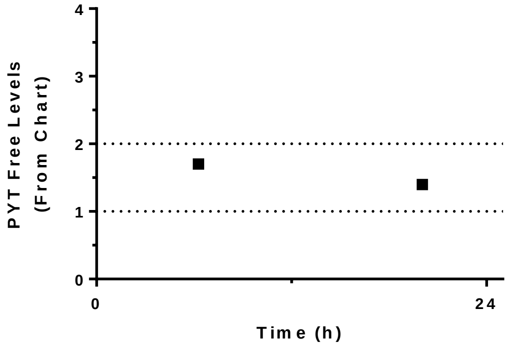
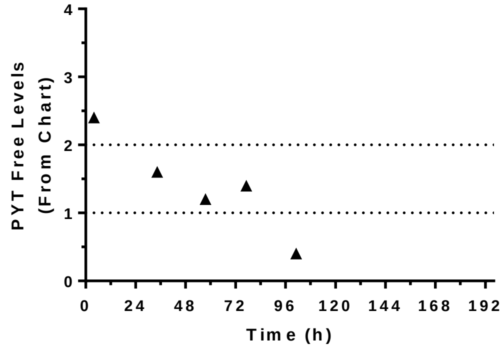
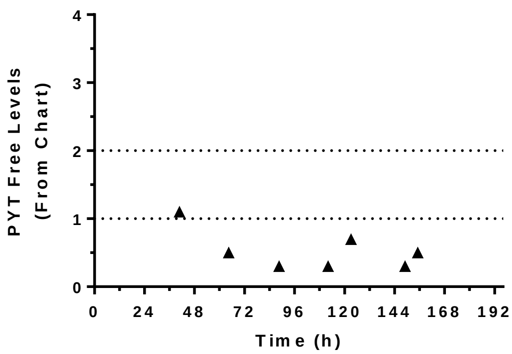
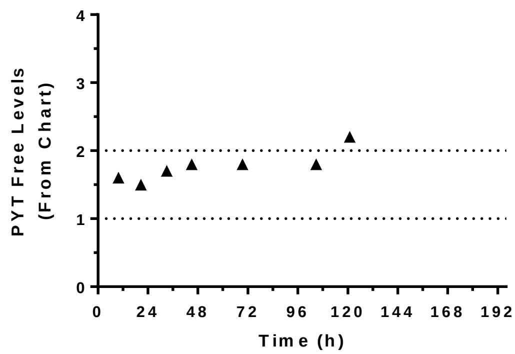
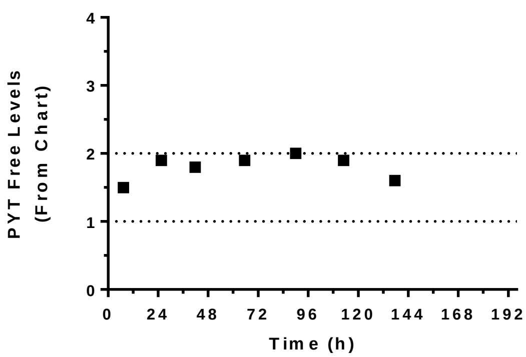
2
3
4
0
24
48
72
96
120
144
Time (h)

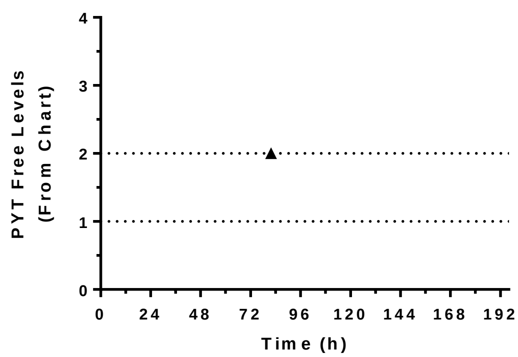

PYT Free Levels
(From Chart)
Time (h)

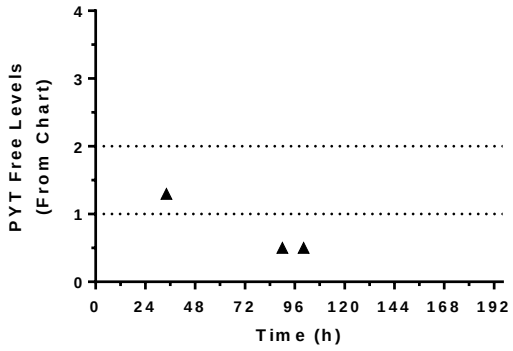

PYT Free Levels
(From Chart)
Time (h)

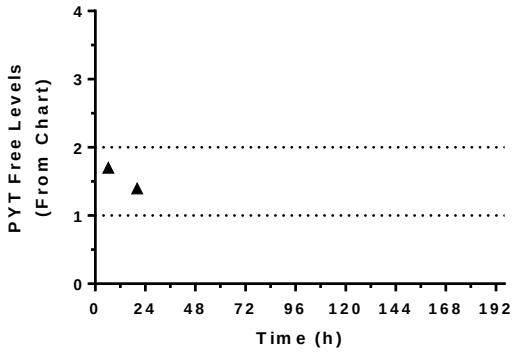

PYT Free Levels
(From Chart)
Time (h)

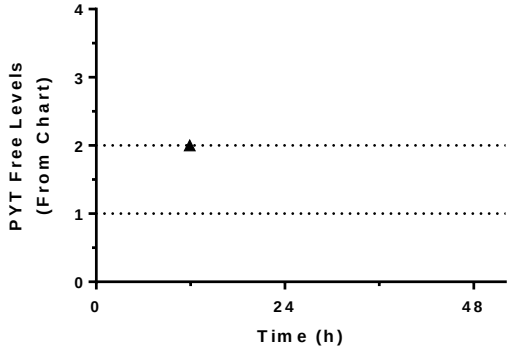

PYT Free Levels
(From Chart)
Time (h)

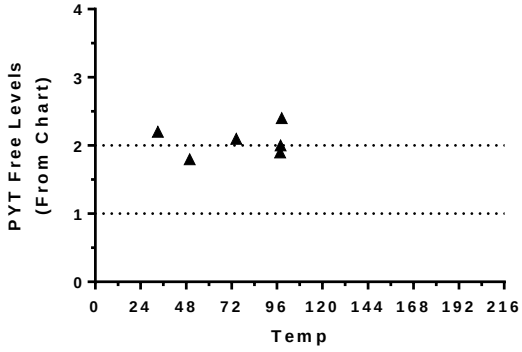

PYT Free Levels
(From Chart)
Time (h)

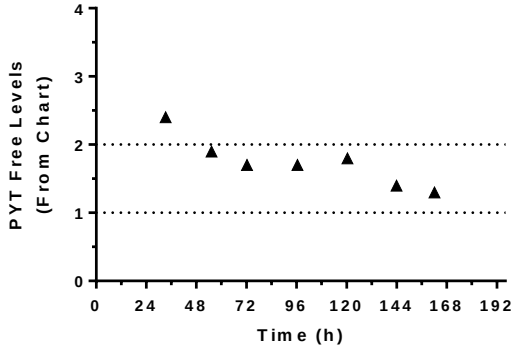

PYT Free Levels
(From Chart)
Time (h)

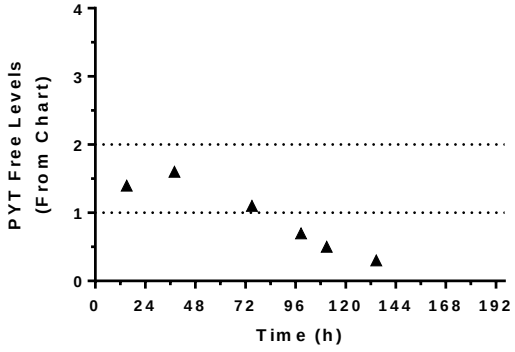

PYT Free Levels
(From Chart)
0
1
2
3
4
0 24 48 72 96 120 144 168 192
Time (h)

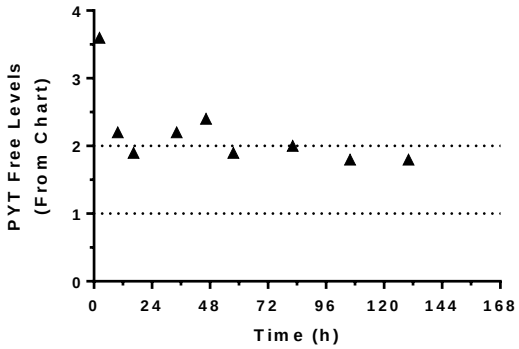

PYT Free Levels
(From Chart)
0
1
2
3
4
0 24 48 72 96 120 144 168 192
Time (h)

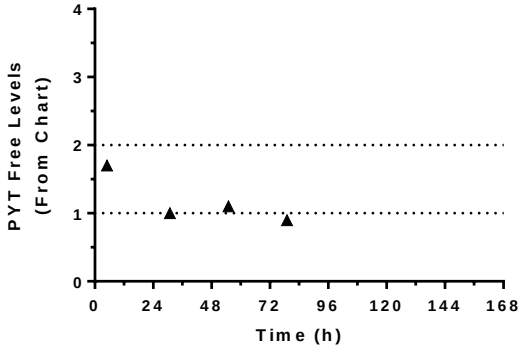

PYT Free Levels
(From Chart)
0
1
2
3
4
0 24 48
Time (h)

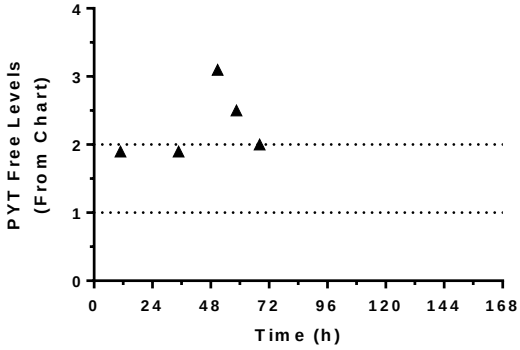

PYT Free Levels
(From Chart)
0
1
2
3
4
0 24 48 72 96 120 144 168 192 216
Temp

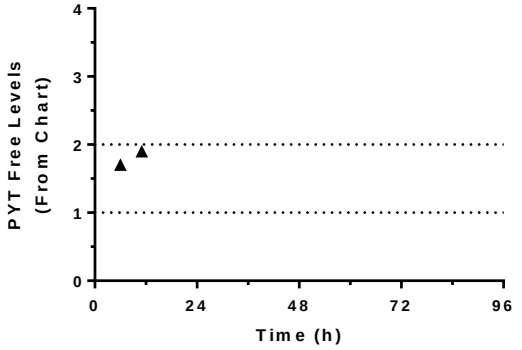

PYT Free Levels
(From Chart)
0
1
2
3
4
0 24 48 72 96 120 144 168 192
Time (h)

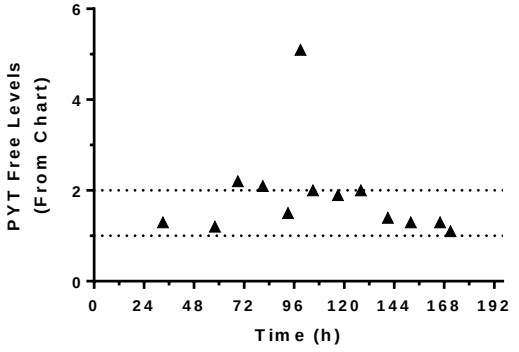

PYT Free Levels
(From Chart)
0
1
2
3
4
0 24 48 72 96 120 144 168 192
Time (h)

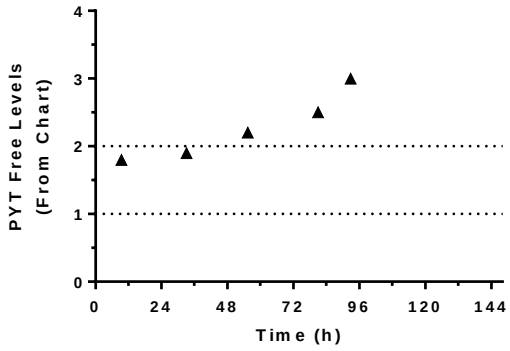

PYT Free Levels
(From Chart)
0
1
2
3
4
0
24
48
72
96
120
144
168
Time (h)

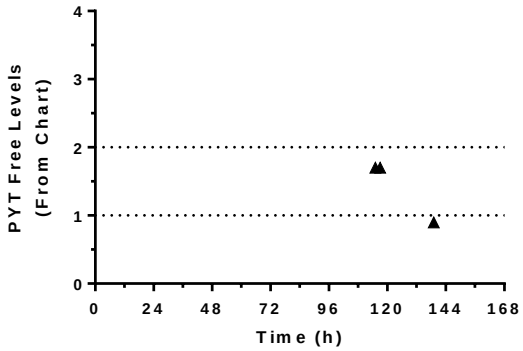

PYT Free Levels
(From Chart)
0
1
2
3
4
0
24
48
72
96
120
144
168
Time (h)

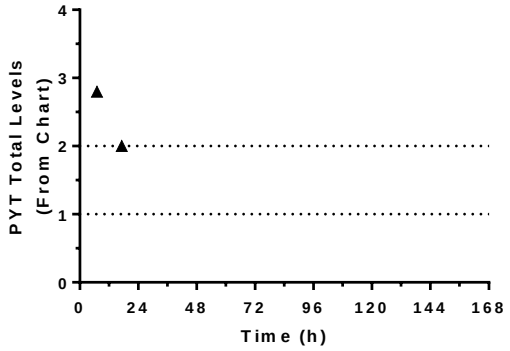

PYT Free Levels
(From Chart)
0
1
2
3
4
0
24
48
72
96
120
144
168
Time (h)

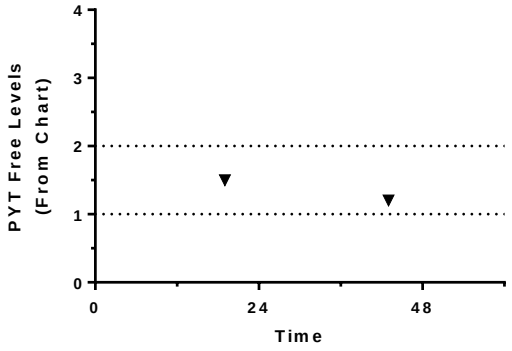

PYT Free Levels
(From Chart)
0
1
2
3
4
0
24
48
72
96
Time (h)


PYT Free Levels
(From Chart)
0
2
4
6
0
24
48
72
96
120
144
168
192
Time (h)


PYT Free Levels
(From Chart)
4
3
2
1
0
0
24
48
72
96
120
144
Time (h)


PYT Free Levels
(From Chart)
4
3
2
1
0
0
24
48
72
96
120
144
168
Time (h)


PYT Total Levels
(From Chart)
4
3
2
1
0
0
24
48
72
96
120
144
168
Time (h)


PYT Free Levels
(From Chart)
4
3
2
1
0
0
24
48
Time

APPENDIX B: INPUT DATA FOR POPULATION PHARMACOKINETIC ANALYSIS

B.1. NONMEM INPUT DATA FILE FOR PHENYTOIN LEVELS IN PEDIATRICS WITH CARDIAC ARREST

The following data depicts the formatted input file for running nonlinear mixed effect modeling (NONMEM) on this data set.

C	ID	TIME	DOSE	AMT	LI_DV	MDV	TOTALCONC	TEMP	FLG_1	WT	GENDER	HT	FLG_CM	LIDV	AGE	DV
.	14	0.1	18.9	680	0	1	0	36	4	36	0	129.5	1	0	8.88	0
.	14	2.3	0	0	3.6	0	30.8	33.4	4	36	0	129.5	1	3.6	8.88	1.280934
.	14	2.3	0	0	30.8	0	30.8	33.4	4	36	0	129.5	2	30.8	8.88	3.427515
.	14	9.8	0	0	2.2	0	14.1	37.5	4	36	0	129.5	1	2.2	8.88	0.788457
.	14	9.8	0	0	14.1	0	14.1	37.5	4	36	0	129.5	2	14.1	8.88	2.646175
.	14	16.4	0	0	1.9	0	12.1	36.2	4	36	0	129.5	1	1.9	8.88	0.641854
.	14	16.4	0	0	12.1	0	12.1	36.2	4	36	0	129.5	2	12.1	8.88	2.493205
.	14	34.2	0	0	2.2	0	13.4	36.8	4	36	0	129.5	1	2.2	8.88	0.788457
.	14	34.2	0	0	13.4	0	13.4	36.8	4	36	0	129.5	2	13.4	8.88	2.595255
.	14	46.3	0	0	2.4	0	13.4	35.5	4	36	0	129.5	1	2.4	8.88	0.875469
.	14	46.3	0	0	13.4	0	13.4	35.5	4	36	0	129.5	2	13.4	8.88	2.595255
.	14	57.6	0	0	1.9	0	13.9	36.6	4	36	0	129.5	1	1.9	8.88	0.641854
.	14	57.6	0	0	13.9	0	13.9	36.6	4	36	0	129.5	2	13.9	8.88	2.631889
.	14	66.2	2.5	90	0	1	0	36.6	4	36	0	129.5	1	0	8.88	0
.	14	77.5	2.5	90	0	1	0	36.6	4	36	0	129.5	1	0	8.88	0
.	14	82.2	0	0	2	0	13.4	37	4	36	0	129.5	1	2	8.88	0.693147
.	14	82.2	0	0	13.4	0	13.4	37	4	36	0	129.5	2	13.4	8.88	2.595255
.	14	89.9	2.5	90	0	1	0	37.3	4	36	0	129.5	1	0	8.88	0
.	14	102.4	2.5	90	0	1	0	36.6	4	36	0	129.5	1	0	8.88	0
.	14	105.9	0	0	1.8	0	14.9	36.3	4	36	0	129.5	1	1.8	8.88	0.587787
.	14	105.9	0	0	14.9	0	14.9	36.3	4	36	0	129.5	2	14.9	8.88	2.701361
.	14	113.8	2.5	90	0	1	0	36.8	4	36	0	129.5	1	0	8.88	0
.	14	127.3	2.5	90	0	1	0	36.6	4	36	0	129.5	1	0	8.88	0
.	14	130	0	0	1.8	0	11.7	36.2	4	36	0	129.5	1	1.8	8.88	0.587787
.	14	130	0	0	11.7	0	11.7	36.2	4	36	0	129.5	2	11.7	8.88	2.459589
.	14	138.5	2.5	90	0	1	0	36.2	4	36	0	129.5	1	0	8.88	0
.	14	149.7	2.5	90	0	1	0	35.6	4	36	0	129.5	1	0	8.88	0
.	15	0.4	20	240	0	1	0	36	1	12	1	.	1	0	8.88	0
.	15	3.1	0	0	20.8	0	20.8	34.1	1	12	1	.	2	20.8	8.88	3.034953
.	15	5	0	0	1.7	0	16.9	30	1	12	1	.	1	1.7	8.88	0.530628
.	15	5	0	0	16.9	0	16.9	30	1	12	1	.	2	16.9	8.88	2.827314
.	15	12.1	2.5	30	0	1	0	33.3	1	12	1	.	1	0	8.88	0
.	15	23.4	2.5	30	0	1	0	32.6	1	12	1	.	1	0	8.88	0
.	15	30.9	0	0	1	0	10	34.5	1	12	1	.	1	1	8.88	0

.	15	30.9	0	0	10	0	10	34.5	1	12	1	.	2	10	8.88	2.302585
.	15	35.5	2.5	30	0	1	0	33.4	1	12	1	.	1	0	8.88	0
.	15	48	2.5	30	0	1	0	32	1	12	1	.	1	0	8.88	0
.	15	54.9	0	0	1.1	0	8.4	35.1	1	12	1	.	1	1.1	8.88	0.09531
.	15	54.9	0	0	8.4	0	8.4	35.1	1	12	1	.	2	8.4	8.88	2.128232
.	15	59.3	2.5	30	0	1	0	34.9	1	12	1	.	1	0	8.88	0
.	15	72.7	2.5	30	0	1	0	36.1	1	12	1	.	1	0	8.88	0
.	15	79.1	0	0	0.9	0	7.1	37	1	12	1	.	1	0.9	8.88	-0.10536
.	15	79.1	0	0	7.1	0	7.1	37	1	12	1	.	2	7.1	8.88	1.960095
.	15	83.7	2.5	30	0	1	0	37.4	1	12	1	.	1	0	8.88	0
.	15	94.9	2.5	30	0	1	0	37	1	12	1	.	1	0	8.88	0
.	15	107.5	2.5	30	0	1	0	37.3	1	12	1	.	1	0	8.88	0
.	15	119.7	2.5	30	0	1	0	36.6	1	12	1	.	1	0	8.88	0
C	15	127.4	0	0	0	0	-99	36	1	12	1	.	1	0	8.88	0
.	15	131.2	2.5	30	0	1	0	36.6	1	12	1	.	1	0	8.88	0
.	16	6.8	20	80	0	1	0	34.9	4	4	0	50	1	0	0.14	0
.	16	10.8	0	0	1.9	0	14.7	35.9	4	4	0	50	1	1.9	0.14	0.641854
.	16	10.8	0	0	14.7	0	14.7	35.9	4	4	0	50	2	14.7	0.14	2.687847
.	16	34.8	0	0	1.9	0	6.4	36.5	4	4	0	50	1	1.9	0.14	0.641854
.	16	34.8	0	0	6.4	0	6.4	36.5	4	4	0	50	2	6.4	0.14	1.856298
.	16	50	20	80	0	1	0	36.6	4	4	0	50	1	0	0.14	0
.	16	50.8	0	0	3.1	0	16.7	36.7	4	4	0	50	1	3.1	0.14	1.131402
.	16	50.8	0	0	16.7	0	16.7	36.7	4	4	0	50	2	16.7	0.14	2.815409
.	16	58.6	0	0	2.5	0	19.7	36. 3	4	4	0	50	1	2.5	0.14	0.916291
.	16	58.6	0	0	19.7	0	19.7	36. 4	4	4	0	50	2	19.7	0.14	2.980619
.	16	68.1	0	0	2	0	14.4	36.6	4	4	0	50	1	2	0.14	0.693147
.	16	68.1	0	0	14.4	0	14.4	36.6	4	4	0	50	2	14.4	0.14	2.667228
.	16	71.9	2.5	10	0	1	0	36.6	4	4	0	50	1	0	0.14	0
.	16	109.3	0	0	9.4	0	9.4	36.6	4	4	0	50	2	9.4	0.14	2.24071
C	22	-0.6	20	140	0	1	0	32.4	2	7	.	72	1	0	.	0
.	22	10.6	3	21	0	1	0	32.4	2	7	.	72	1	0	.	0
.	22	12	0	0	1.3	0	10.7	33	2	7	.	72	1	1.3	.	0.262364
.	22	12	0	0	10.7	0	10.7	33	2	7	.	72	2	10.7	.	2.370244
.	22	22.7	3	21	0	1	0	32.3	2	7	.	72	1	0	.	0

.	22	34.3	3	21	0	1	0	32.5	2	7	.	72	1	0	.	0
.	22	35.3	0	0	1.1	0	0	32.4	2	7	.	72	1	1.1	.	0.09531
.	22	35.3	0	0	9.6	0	0	32.4	2	7	.	72	2	9.6	.	2.261763
.	22	45	3	21	0	1	0	32.3	2	7	.	72	1	0	.	0
.	22	58.3	3	21	0	1	0	33.1	2	7	.	72	1	0	.	0
.	22	60	0	0	1.1	0	8.8	32.8	2	7	.	72	1	1.1	.	0.09531
.	22	60	0	0	8.8	0	8.8	32.8	2	7	.	72	2	8.8	.	2.174752
.	22	70	3	21	0	1	0	34.8	2	7	.	72	1	0	.	0
.	22	82	3	21	0	1	0	36	2	7	.	72	1	0	.	0
.	22	85.2	0	0	0.8	0	7	35.6	2	7	.	72	1	0.8	.	-0.22314
.	22	85.2	0	0	7	0	7	35.6	2	7	.	72	2	7	.	1.94591
.	22	95.3	3	21	0	1	0	36	2	7	.	72	1	0	.	0
.	22	107.4	3	21	0	1	0	36.6	2	7	.	72	1	0	.	0
.	22	108.4	0	0	1	0	10.1	36.5	2	7	.	72	1	1	.	0
.	22	108.4	0	0	10.1	0	10.1	36.5	2	7	.	72	2	10.1	.	2.312535
.	22	119.3	3	21	0	1	0	36.6	2	7	.	72	1	0	.	0
.	22	131.5	3	21	0	1	0	35.6	2	7	.	72	1	0	.	0
.	22	132.5	0	0	0.8	0	6.5	35.9	2	7	.	72	1	0.8	.	-0.22314
.	22	132.5	0	0	6.5	0	6.5	35.9	2	7	.	72	2	6.5	.	1.871802
.	22	144.3	3	21	0	1	0	34.9	2	7	.	72	1	0	.	0
.	23	2.2	20.6	330	0	1	0	32.3	5	16	0	87.5	1	0	3.93	0
.	23	5.4	0	0	2.6	0	20.3	36.2	5	16	0	87.5	1	2.6	3.93	0.955511
.	23	5.4	0	0	20.3	0	20.3	36.2	5	16	0	87.5	2	20.3	3.93	3.010621
.	23	7.2	2.6	42	0	1	0	36.7	5	16	0	87.5	1	0	3.93	0
.	23	9	0	0	2.6	0	15.6	36	5	16	0	87.5	1	2.6	3.93	0.955511
.	23	9	0	0	15.6	0	15.6	36	5	16	0	87.5	2	15.6	3.93	2.747271
.	23	16.8	2.6	42	0	1	0	37.6	5	16	0	87.5	1	0	3.93	0
.	23	36.7	0	0	1.6	0	13.1	36.8	5	16	0	87.5	1	1.6	3.93	0.470004
.	23	36.7	0	0	13.1	0	13.1	36.8	5	16	0	87.5	2	13.1	3.93	2.572612
.	23	61	0	0	1.3	0	0	36	5	16	0	87.5	1	1.3	3.93	0.262364
.	23	61	0	0	11.7	0	0	36	5	16	0	87.5	2	11.7	3.93	2.459589
.	24	1.3	21.3	700	0	1	0	34.2	2	32.8	0	.	1	0	3.93	0
.	24	12.5	0	0	1.5	0	10.7	32.9	2	32.8	0	.	1	1.5	3.93	0.405465
.	24	12.5	0	0	10.7	0	10.7	32.9	2	32.8	0	.	2	10.7	3.93	2.370244

.	24	14	3	105	0	1	0	30.6	2	32.8	0	.	1	0	3.93	0
.	24	25.8	2.9	105	0	1	0	30.4	2	32.8	0	.	1	0	3.93	0
.	24	37	2.9	105	0	1	0	33.4	2	32.8	0	.	1	0	3.93	0
.	24	37	0	0	1.8	0	10.9	33.4	2	32.8	0	.	1	1.8	3.93	0.587787
.	24	37	0	0	10.9	0	10.9	33.4	2	32.8	0	.	2	10.9	3.93	2.388763
.	24	49.3	2.8	105	0	1	0	31.7	2	32.8	0	.	1	0	3.93	0
.	25	0	19.2	300	0	1	0	38.9	5	15.6	0	85	1	0	3.37	0
.	25	11.3	0	0	1.2	0	12.6	37.8	5	15.6	0	85	1	1.2	3.37	0.182322
.	25	11.3	0	0	12.6	0	12.6	37.8	5	15.6	0	85	2	12.6	3.37	2.533697
.	25	12.6	2.4	37	0	1	0	37.6	5	15.6	0	85	1	0	3.37	0
.	25	24	2.4	37	0	1	0	37.4	5	15.6	0	85	1	0	3.37	0
.	25	35	0	0	1.7	0	13	36.9	5	15.6	0	85	1	1.7	3.37	0.530628
.	25	35	0	0	13	0	13	36.9	5	15.6	0	85	2	13	3.37	2.564949
.	25	36.8	2.4	37	0	1	0	36.9	5	15.6	0	85	1	0	3.37	0
.	25	49	2.4	37	0	1	0	36.6	5	15.6	0	85	1	0	3.37	0
.	25	55	0	0	1.9	0	12.5	35.8	5	15.6	0	85	1	1.9	3.37	0.641854
.	25	55	0	0	12.5	0	12.5	35.8	5	15.6	0	85	2	12.5	3.37	2.525729
.	25	61.9	2.4	37	0	1	0	37.1	5	15.6	0	85	1	0	3.37	0
.	25	74.2	2.4	37	0	1	0	36.3	5	15.6	0	85	1	0	3.37	0
.	25	79	0	0	1.2	0	8.4	36.4	5	15.6	0	85	1	1.2	3.37	0.182322
.	25	79	0	0	8.4	0	8.4	36.4	5	15.6	0	85	2	8.4	3.37	2.128232
.	25	86.2	2.4	37	0	1	0	36.3	5	15.6	0	85	1	0	3.37	0
.	25	97.7	2.4	37	0	1	0	36.9	5	15.6	0	85	1	0	3.37	0
.	25	102.8	0	0	0.4	0	3.7	36.4	5	15.6	0	85	1	0.4	3.37	-0.91629
.	25	102.8	0	0	3.7	0	3.7	36.4	5	15.6	0	85	2	3.7	3.37	1.308333
.	25	109.9	2.6	40	0	1	0	37.1	5	15.6	0	85	1	0	3.37	0
.	25	122.3	2.6	40	0	1	0	37	5	15.6	0	85	1	0	3.37	0
.	25	131.3	0	0	0.5	0	2.7	36.5	5	15.6	0	85	1	0.5	3.37	-0.69315
.	25	131.3	0	0	2.7	0	2.7	36.5	5	15.6	0	85	2	2.7	3.37	0.993252
.	25	134.3	2.6	40	0	1	0	37.7	5	15.6	0	85	1	0	3.37	0
.	25	145.4	2.6	40	0	1	0	37.5	5	15.6	0	85	1	0	3.37	0
.	26	6.2	17.1	300	0	1	0	31	2	17.5	.	102	1	0	2.31	0
.	26	9.9	0	0	2.3	0	15.5	33	2	17.5	.	102	1	2.3	2.31	0.832909
.	26	9.9	0	0	15.5	0	15.5	33	2	17.5	.	102	2	15.5	2.31	2.74084

.	26	13.8	0	0	2.1	0	16.1	32.6	2	17.5	.	102	1	2.1	2.31	0.741937
.	26	13.8	0	0	16.1	0	16.1	32.6	2	17.5	.	102	2	16.1	2.31	2.778819
.	26	18	0	0	17.4	0	17.4	33.4	2	17.5	.	102	2	17.4	2.31	2.85647
.	26	31.3	2.3	40	0	1	0	33.3	2	17.5	.	102	1	0	2.31	0
.	26	39.2	0	0	1.6	0	0	33.3	2	17.5	.	102	1	1.6	2.31	0.470004
.	26	39.2	0	0	14.9	0	0	33.3	2	17.5	.	102	2	14.9	2.31	2.701361
.	26	42	2.3	40	0	1	0	33.1	2	17.5	.	102	1	0	2.31	0
.	26	56.4	2.3	40	0	1	0	33.3	2	17.5	.	102	1	0	2.31	0
.	26	66.3	0	0	1.7	0	16	34.6	2	17.5	.	102	1	1.7	2.31	0.530628
.	26	66.3	0	0	16	0	16	34.6	2	17.5	.	102	2	16	2.31	2.772589
.	26	67.8	2.3	40	0	1	0	35.1	2	17.5	.	102	1	0	2.31	0
.	26	80.7	2.3	40	0	1	0	37.1	2	17.5	.	102	1	0	2.31	0
.	26	108.3	0	0	17.1	0	17.1	36.8	2	17.5	.	102	2	17.1	2.31	2.839078
C	17	.	2.1	20	0	1	0	37.6	4	9.6	0	74	1	0	0.95	0
.	17	5	0	0	1.7	0	12.5	37.6	4	9.6	0	74	1	1.7	0.95	0.530628
.	17	5	0	0	12.5	0	12.5	37.6	4	9.6	0	74	2	12.5	0.95	2.525729
.	17	8.5	3.1	30	0	1	0	37.9	4	9.6	0	74	1	0	0.95	0
.	17	10	0	0	1.9	0	18.5	37.4	4	9.6	0	74	1	1.9	0.95	0.641854
.	17	10	0	0	18.5	0	18.5	37.4	4	9.6	0	74	2	18.5	0.95	2.917771
.	17	18.5	3.1	30	0	1	0	37.5	4	9.6	0	74	1	0	0.95	0
C	18	-0.3	0	240	0	1	0	32.4	.	13	0	89	1	0	3.53	0
.	18	1.8	0	0	4.3	0	8.5	32.1	.	13	0	89	1	4.3	3.53	1.458615
.	18	1.8	0	0	8.5	0	8.5	32.1	.	13	0	89	2	8.5	3.53	2.140066
.	18	10.9	0	0	8.4	0	8.4	35.8	.	13	0	89	2	8.4	3.53	2.128232
.	20	93	20	360	0	1	0	38	4	18	0	105	1	0	7.79	0
.	20	94	0	0	3.1	0	23.9	38	4	18	0	105	1	3.1	7.79	1.131402
.	20	94	0	0	23.9	0	23.9	38	4	18	0	105	2	23.9	7.79	3.173878
.	20	111	0	0	1.5	0	10	37.8	4	18	0	105	1	1.5	7.79	0.405465
.	20	111	0	0	10	0	10	37.8	4	18	0	105	2	10	7.79	2.302585
.	20	121	0	0	0.9	0	5.7	37.2	4	18	0	105	1	0.9	7.79	-0.10536
.	20	121	0	0	5.7	0	5.7	37.2	4	18	0	105	2	5.7	7.79	1.740466
.	20	123	5	90	0	1	0	37.6	4	18	0	105	1	0	7.79	0
.	20	126	5	90	0	1	0	37.6	4	18	0	105	1	0	7.79	0
.	20	130	0	0	2	0	13.8	37.6	4	18	0	105	1	2	7.79	0.693147

.	20	130	0	0	13.8	0	13.8	37.6	4	18	0	105	2	13.8	7.79	2.624669
.	20	133	0	0	1.7	0	11.3	37.6	4	18	0	105	1	1.7	7.79	0.530628
.	20	133	0	0	11.3	0	11.3	37.6	4	18	0	105	2	11.3	7.79	2.424803
.	20	134	3.3	60	0	1	0	37.8	4	18	0	105	1	0	7.79	0
.	20	147	3.3	60	0	1	0	37.2	4	18	0	105	1	0	7.79	0
.	20	147	0	0	0.8	0	6.1	37.2	4	18	0	105	1	0.8	7.79	-0.22314
.	20	147	0	0	6.1	0	6.1	37.2	4	18	0	105	2	6.1	7.79	1.808289
.	20	149	10	180	0	1	0	37.6	4	18	0	105	1	0	7.79	0
.	20	150	0	0	2.6	0	16.9	37.2	4	18	0	105	1	2.6	7.79	0.955511
.	20	150	0	0	16.9	0	16.9	37.2	4	18	0	105	2	16.9	7.79	2.827314
.	20	157	0	0	11.7	0	11.7	37.4	4	18	0	105	2	11.7	7.79	2.459589
.	20	157.7	0	0	11.7	0	11.7	37.4	4	18	0	105	2	11.7	7.79	2.459589
.	20	159	0	0	1.4	0	9.4	36.8	4	18	0	105	1	1.4	7.79	0.336472
.	20	159	0	0	9.4	0	9.4	36.8	4	18	0	105	2	9.4	7.79	2.24071
.	20	162	3.7	66	0	1	0	37.2	4	18	0	105	1	0	7.79	0
.	20	174	3.7	66	0	1	0	36.8	4	18	0	105	1	0	7.79	0
.	27	1.1	2.5	150	0	1	0	35	3	60	1	170.2	1	0	16.63	0
.	27	1.3	20	1200	0	1	0	35.1	3	60	1	170.2	1	0	16.63	0
.	27	13.5	2.5	150	0	1	0	32.2	3	60	1	170.2	1	0	16.63	0
.	27	15	0	0	2.4	0	23.1	33	3	60	1	170.2	1	2.4	16.63	0.875469
.	27	15	0	0	23.1	0	23.1	33	3	60	1	170.2	2	23.1	16.63	3.139833
.	27	24.2	0	0	2.3	0	22.2	33.2	3	60	1	170.2	1	2.3	16.63	0.832909
.	27	24.2	0	0	22.2	0	22.2	33.2	3	60	1	170.2	2	22.2	16.63	3.100092
.	27	24.9	2.5	150	0	1	0	33.9	3	60	1	170.2	1	0	16.63	0
.	27	35.3	0	0	2.4	0	24.4	34.3	3	60	1	170.2	1	2.4	16.63	0.875469
.	27	35.3	0	0	24.4	0	24.4	34.3	3	60	1	170.2	2	24.4	16.63	3.194583
.	27	44.5	0	0	2.3	0	23.9	35.4	3	60	1	170.2	1	2.3	16.63	0.832909
.	27	44.5	0	0	23.9	0	23.9	35.4	3	60	1	170.2	2	23.9	16.63	3.173878
.	27	64.4	0	0	2.1	0	19.9	37.4	3	60	1	170.2	1	2.1	16.63	0.741937
.	27	64.4	0	0	19.9	0	19.9	37.4	3	60	1	170.2	2	19.9	16.63	2.99072
.	27	87.3	0	0	2	0	17.7	37.8	3	60	1	170.2	1	2	16.63	0.693147
.	27	87.3	0	0	17.7	0	17.7	37.8	3	60	1	170.2	2	17.7	16.63	2.873565
.	27	93.3	0	0	1.9	0	16.3	38	3	60	1	170.2	1	1.9	16.63	0.641854
.	27	93.3	0	0	16.3	0	16.3	38	3	60	1	170.2	2	16.3	16.63	2.791165

.	27	111.3	0	0	1.3	0	12.9	37.8	3	60	1	170.2	1	1.3	16.63	0.262364
.	27	111.3	0	0	12.9	0	12.9	37.8	3	60	1	170.2	2	12.9	16.63	2.557227
.	27	123.2	0	0	1	0	9.8	38.4	3	60	1	170.2	1	1	16.63	0
.	27	123.2	0	0	9.8	0	9.8	38.4	3	60	1	170.2	2	9.8	16.63	2.282382
.	27	135	0	0	0.7	0	6.5	38.2	3	60	1	170.2	1	0.7	16.63	-0.35667
.	27	135	0	0	6.5	0	6.5	38.2	3	60	1	170.2	2	6.5	16.63	1.871802
C	28	-1.3	20	100	0	.	0	.	1	.	.	.	1	0	0.15	0
.	28	3.9	2	10	0	1	0	33.1	1	5	1	.	1	0	0.15	0
.	28	9.7	0	0	1.2	0	7.6	33.3	1	5	1	.	1	1.2	0.15	0.182322
.	28	9.7	0	0	7.6	0	7.6	33.3	1	5	1	.	2	7.6	0.15	2.028148
.	28	14.2	2	10	0	1	0	33.5	1	5	1	.	1	0	0.15	0
.	28	30	0	0	0.5	0	3.9	35.7	1	5	1	.	1	0.5	0.15	-0.69315
.	28	30	0	0	3.9	0	3.9	35.7	1	5	1	.	2	3.9	0.15	1.360977
.	29	1	12	1000	0	1	0	34.2	1	83.5	1	.	1	0	.	0
.	29	6	0	0	1.7	0	10.3	33.8	1	83.5	1	.	1	1.7	.	0.530628
.	29	6	0	0	10.3	0	10.3	33.8	1	83.5	1	.	2	10.3	.	2.332144
.	29	12	0	0	1.5	0	10.4	33.8	1	83.5	1	.	1	1.5	.	0.405465
.	29	12	0	0	10.4	0	10.4	33.8	1	83.5	1	.	2	10.4	.	2.341806
.	29	13	2.4	200	0	1	0	33.8	1	83.5	1	.	1	0	.	0
.	29	25.9	2.4	200	0	1	0	35.6	1	83.5	1	.	1	0	.	0
.	29	36	0	0	2.2	0	15.7	37.1	1	83.5	1	.	1	2.2	.	0.788457
.	29	36	0	0	15.7	0	15.7	37.1	1	83.5	1	.	2	15.7	.	2.753661
.	29	36.6	2.4	200	0	1	0	37.1	1	83.5	1	.	1	0	.	0
.	30	0.3	24	600	0	1	0	33.3	1	25	0	122	1	0	8.79	0
.	30	1.7	0	0	15.9	0	15.9	33.2	1	25	0	122	2	15.9	8.79	2.766319
.	30	4.8	0	0	4.6	0	15.9	37.4	1	25	0	122	1	4.6	8.79	1.526056
.	30	4.8	0	0	15.9	0	15.9	37.4	1	25	0	122	2	15.9	8.79	2.766319
.	30	11	0	0	2	0	10.8	33	1	25	0	122	1	2	8.79	0.693147
.	30	11	0	0	10.8	0	10.8	33	1	25	0	122	2	10.8	8.79	2.379546
.	30	12	3	75	0	1	0	34.4	1	25	0	122	1	0	8.79	0
.	30	24	3	75	0	1	0	36.2	1	25	0	122	1	0	8.79	0
.	30	34.8	0	0	2.3	0	15.6	35.4	1	25	0	122	1	2.3	8.79	0.832909
.	30	34.8	0	0	15.6	0	15.6	35.4	1	25	0	122	2	15.6	8.79	2.747271
.	30	58.9	0	0	2.2	0	14.3	36.6	1	25	0	122	1	2.2	8.79	0.788457

.	30	58.9	0	0	14.3	0	14.3	36.6	1	25	0	122	2	14.3	8.79	2.66026
.	30	114.2	0	0	1.3	0	8.3	37.4	1	25	0	122	1	1.3	8.79	0.262364
.	30	114.2	0	0	8.3	0	8.3	37.4	1	25	0	122	2	8.3	8.79	2.116256
C	31	-2.1	20	200	0	1	0	36.4	5	9	0	.	1	0	1.42	0
.	31	5	0	0	2.3	0	17	37	5	10	0	.	1	2.3	1.42	0.832909
.	31	5	0	0	17	0	17	37	5	10	0	.	2	17	1.42	2.833213
.	31	9.3	2.5	25	0	1	0	37	5	10	0	.	1	0	1.42	0
.	31	13.1	0	0	2.1	0	14.7	37.1	5	10	0	.	1	2.1	1.42	0.741937
.	31	13.1	0	0	14.7	0	14.7	37.1	5	10	0	.	2	14.7	1.42	2.687847
.	31	19	2.5	25	0	1	0	36.9	5	10	0	.	1	0	1.42	0
.	31	31	2.5	25	0	1	0	37.3	5	10	0	.	1	0	1.42	0
.	31	36.7	0	0	2.2	0	14.3	36.9	5	10	0	.	1	2.2	1.42	0.788457
.	31	36.7	0	0	14.3	0	14.3	36.9	5	10	0	.	2	14.3	1.42	2.66026
.	31	60.9	0	0	1.1	0	6.6	38.3	5	10	0	.	1	1.1	1.42	0.09531
.	31	60.9	0	0	6.6	0	6.6	38.3	5	10	0	.	2	6.6	1.42	1.88707
.	31	82.4	20	200	0	1	0	37.3	5	10	0	.	1	0	1.42	0
.	31	85	0	0	4.1	0	21.9	36.6	5	10	0	.	1	4.1	1.42	1.410987
.	31	85	0	0	21.9	0	21.9	36.6	5	10	0	.	2	21.9	1.42	3.086487
.	36	-3.5	0	80	0	1	0	37.8	.	.	.	.	1	0	.	0
.	36	35.3	0	1900	0	1	0	37.4	.	.	.	.	1	0	.	0
.	36	37.5	0	0	2.4	0	11.4	37.6	.	.	.	.	1	2.4	.	0.875469
.	36	37.5	0	0	11.4	0	11.4	37.6	.	.	.	.	2	11.4	.	2.433613
.	36	45	0	0	2.5	0	11.6	37.4	.	.	.	.	1	2.5	.	0.916291
.	36	45	0	0	11.6	0	11.6	37.4	.	.	.	.	2	11.6	.	2.451005
.	36	69.5	0	0	2.3	0	9.1	37	.	.	.	.	1	2.3	.	0.832909
.	36	69.5	0	0	9.1	0	9.1	37	.	.	.	.	2	9.1	.	2.208274
.	8	23.9	20	240	0	1	0	33	1	12	0	80	1	0	2.44	0
.	8	27.1	0	0	16.3	0	16.3	33	1	12	0	80	2	16.3	2.44	2.791165
.	8	30.6	5	60	0	1	0	33.4	1	12	0	80	1	0	2.44	0
.	8	34.1	0	0	1.3	0	16.8	34	1	12	0	80	1	1.3	2.44	0.262364
.	8	34.1	0	0	16.8	0	16.8	34	1	12	0	80	2	16.8	2.44	2.821379
.	8	36.5	2.5	30	0	1	0	34.6	1	12	0	80	1	0	2.44	0
.	8	46.5	2.5	30	0	1	0	36.7	1	12	0	80	1	0	2.44	0
.	8	58.1	2.5	30	0	1	0	37	1	12	0	80	1	0	2.44	0

.	8	70.1	2.5	30	0	1	0	36.6	1	12	0	80	1	0	2.44	0
.	8	82.4	2.5	30	0	1	0	38	1	12	0	80	1	0	2.44	0
.	8	90.1	0	0	0.5	0	6.1	37.6	1	12	0	80	1	0.5	2.44	-0.69315
.	8	90.1	0	0	6.1	0	6.1	37.6	1	12	0	80	2	6.1	2.44	1.808289
.	8	95	5	60	0	1	0	38.2	1	12	0	80	1	0	2.44	0
.	8	100.3	0	0	0.5	0	6.1	37.7	1	12	0	80	1	0.5	2.44	-0.69315
.	8	100.3	0	0	6.1	0	6.1	37.7	1	12	0	80	2	6.1	2.44	1.808289
.	8	106.2	2.8	33	0	1	0	37.3	1	12	0	80	1	0	2.44	0
.	8	118.9	2.8	33	0	1	0	38.3	1	12	0	80	1	0	2.44	0
.	8	121.3	5	60	0	1	0	38.2	1	12	0	80	1	0	2.44	0
.	8	130.4	3	36	0	1	0	39	1	12	0	80	1	0	2.44	0
.	8	145.1	3	36	0	1	0	38.5	1	12	0	80	1	0	2.44	0
.	8	154.7	3	36	0	1	0	37.9	1	12	0	80	1	0	2.44	0
.	11	28.5	20.4	1200	0	1	0	34.9	3	58.9	0	154.9	1	0	10.36	0
.	11	33	0	0	2.2	0	20.7	32.6	3	58.9	0	154.9	1	2.2	10.36	0.788457
.	11	33	0	0	20.7	0	20.7	32.6	3	58.9	0	154.9	2	20.7	10.36	3.030134
.	11	38.5	2.6	150	0	1	0	34	3	58.9	0	154.9	1	0	10.36	0
.	11	46	2.6	150	0	1	0	33.4	3	58.9	0	154.9	1	0	10.36	0
.	11	49.9	0	0	1.8	0	16.4	34	3	58.9	0	154.9	1	1.8	10.36	0.587787
.	11	49.9	0	0	16.4	0	16.4	34	3	58.9	0	154.9	2	16.4	10.36	2.797281
.	11	58	2.6	150	0	1	0	32.3	3	58.9	0	154.9	1	0	10.36	0
.	11	70	2.6	150	0	1	0	32.2	3	58.9	0	154.9	2	0	10.36	0
.	11	74.5	0	0	2.1	0	16.5	33.3	3	58.9	0	154.9	1	2.1	10.36	0.741937
.	11	74.5	0	0	16.5	0	16.5	33.3	3	58.9	0	154.9	2	16.5	10.36	2.80336
.	11	82.3	2.6	150	0	1	0	34.6	3	58.9	0	154.9	1	0	10.36	0
.	11	94.8	2.6	150	0	1	0	34.4	3	58.9	0	154.9	1	0	10.36	0
.	11	97.6	0	0	1.9	0	18.5	34	3	58.9	0	154.9	1	1.9	10.36	0.641854
.	11	97.6	0	0	18.5	0	18.5	34	3	58.9	0	154.9	2	18.5	10.36	2.917771
.	11	97.9	0	0	2	0	14.4	34	3	58.9	0	154.9	1	2	10.36	0.693147
.	11	97.9	0	0	14.4	0	14.4	34	3	58.9	0	154.9	2	14.4	10.36	2.667228
.	11	98.5	0	0	2.4	0	18.4	34.1	3	58.9	0	154.9	1	2.4	10.36	0.875469
.	11	98.5	0	0	18.4	0	18.4	34.1	3	58.9	0	154.9	2	18.4	10.36	2.912351
.	11	106.4	2.6	150	0	1	0	36.1	3	58.9	0	154.9	1	0	10.36	0
.	11	118.6	2.6	150	0	1	0	37.5	3	58.9	0	154.9	1	0	10.36	0

.	11	131.4	2.6	150	0	1	0	36.8	3	58.9	0	154.9	1	0	10.36	0
.	11	141.1	2.6	150	0	1	0	37	3	58.9	0	154.9	1	0	10.36	0
.	11	154.3	2.6	150	0	1	0	37.6	3	58.9	0	154.9	1	0	10.36	0
.	1	4	20	100	0	1	0	32.4	1	5	1	.	1	0	0.15	0
.	1	6.3	0	0	1.7	0	12.6	32.7	1	5	1	.	1	1.7	0.15	0.530628
.	1	6.3	0	0	12.6	0	12.6	32.7	1	5	1	.	2	12.6	0.15	2.533697
.	1	16.2	2.5	12.5	0	1	0	33.1	1	5	1	.	1	0	0.15	0
.	1	20.1	0	0	1.4	0	9	33.2	1	5	1	.	1	1.4	0.15	0.336472
.	1	20.1	0	0	9	0	9	33.2	1	5	1	.	2	9	0.15	2.197225
.	2	0	20	300	0	1	0	33.6	1	15	1	92	1	0	3.36	0
.	2	4	0	0	2.4	0	20.6	35.6	1	15	1	92	1	2.4	3.36	0.875469
.	2	4	0	0	20.6	0	20.6	35.6	1	15	1	92	2	20.6	3.36	3.025291
.	2	13.7	3	45	0	1	0	33.7	1	15	1	92	1	0	3.36	0
.	2	24.9	3	45	0	1	0	33.7	1	15	1	92	1	0	3.36	0
.	2	34.3	0	0	1.6	0	14.7	34.4	1	15	1	92	1	1.6	3.36	0.470004
.	2	34.3	0	0	14.7	0	14.7	34.4	1	15	1	92	2	14.7	3.36	2.687847
.	2	37.9	3	45	0	1	0	34.5	1	15	1	92	1	0	3.36	0
.	2	50.6	3	45	0	1	0	36.8	1	15	1	92	1	0	3.36	0
.	2	57.5	0	0	1.2	0	9.7	37.1	1	15	1	92	1	1.2	3.36	0.182322
.	2	57.5	0	0	9.7	0	9.7	37.1	1	15	1	92	2	9.7	3.36	2.272126
.	2	62.5	3	45	0	1	0	37.1	1	15	1	92	1	0	3.36	0
.	2	72.8	3	45	0	1	0	37.3	1	15	1	92	1	0	3.36	0
.	2	77.2	0	0	1.4	0	10	36.9	1	15	1	92	1	1.4	3.36	0.336472
.	2	77.2	0	0	10	0	10	36.9	1	15	1	92	2	10	3.36	2.302585
.	2	85.3	3	45	0	1	0	38.2	1	15	1	92	1	0	3.36	0
.	2	101.1	0	0	0.4	0	3.1	36.2	1	15	1	92	1	0.4	3.36	-0.91629
.	2	101.1	0	0	3.1	0	3.1	36.2	1	15	1	92	2	3.1	3.36	1.131402
.	2	134.3	10	150	0	1	0	36.5	1	15	1	92	1	0	3.36	0
.	2	158.3	3	45	0	1	0	36	1	15	1	92	1	0	3.36	0
.	3	3	20	250	0	1	0	34.4	1	12.5	0	88	1	0	1.55	0
.	3	5	2.5	31	0	1	0	37.2	1	12.5	0	88	1	0	1.55	0
.	3	17	2.5	31	0	1	0	34.9	1	12.5	0	88	1	0	1.55	0
.	3	17	0	0	16	0	16	34.9	1	12.5	0	88	2	16	1.55	2.772589
.	3	29.6	2.5	31	0	1	0	32.7	1	12.5	0	88	1	0	1.55	0

.	3	40.7	0	0	1.1	0	13	34.5	1	12.5	0	88	1	1.1	1.55	0.09531
.	3	40.7	0	0	13	0	13	34.5	1	12.5	0	88	2	13	1.55	2.564949
.	3	41.4	2.5	31	0	1	0	34.5	1	12.5	0	88	1	0	1.55	0
.	3	53	2.5	31	0	1	0	36.7	1	12.5	0	88	1	0	1.55	0
.	3	64.4	0	0	0.5	0	9.6	37.3	1	12.5	0	88	1	0.5	1.55	-0.69315
.	3	64.4	0	0	9.6	0	9.6	37.3	1	12.5	0	88	2	9.6	1.55	2.261763
.	3	64.6	2.5	31	0	1	0	37.2	1	12.5	0	88	1	0	1.55	0
.	3	78.5	2.5	31	0	1	0	37.3	1	12.5	0	88	1	0	1.55	0
.	3	88.6	0	0	0.3	0	7.4	36.5	1	12.5	0	88	1	0.3	1.55	-1.20397
.	3	88.6	0	0	7.4	0	7.4	36.5	1	12.5	0	88	2	7.4	1.55	2.00148
.	3	88.6	2.8	35	0	1	0	36.5	1	12.5	0	88	1	0	1.55	0
.	3	102	2.8	35	0	1	0	37.7	1	12.5	0	88	1	0	1.55	0
.	3	112.1	0	0	0.3	0	4.2	36.4	1	12.5	0	88	1	0.3	1.55	-1.20397
.	3	112.1	0	0	4.2	0	4.2	36.4	1	12.5	0	88	2	4.2	1.55	1.435085
.	3	121.2	4.8	60	0	1	0	37	1	12.5	0	88	1	0	1.55	0
.	3	123.1	0	0	0.7	0	8.7	37.1	1	12.5	0	88	1	0.7	1.55	-0.35667
.	3	123.1	0	0	8.7	0	8.7	37.1	1	12.5	0	88	2	8.7	1.55	2.163323
.	3	127.4	3.4	42	0	1	0	35	1	12.5	0	88	1	0	1.55	0
.	3	139.3	4	50	0	1	0	36.5	1	12.5	0	88	1	0	1.55	0
.	3	144.9	0	0	5.3	0	5.3	36.8	1	12.5	0	88	2	5.3	1.55	1.667707
.	3	149	0	0	0.3	0	3.2	36.8	1	12.5	0	88	1	0.3	1.55	-1.20397
.	3	149	0	0	3.2	0	3.2	36.8	1	12.5	0	88	2	3.2	1.55	1.163151
.	3	151.4	4	50	0	1	0	36.2	1	12.5	0	88	1	0	1.55	0
.	3	155.1	0	0	0.5	0	5.9	35.6	1	12.5	0	88	1	0.5	1.55	-0.69315
.	3	155.1	0	0	5.9	0	5.9	35.6	1	12.5	0	88	2	5.9	1.55	1.774952
.	5	2.8	18.8	1000	0	1	0	37.5	3	53.2	0	161.5	1	0	16.27	0
.	5	9.8	0	0	1.6	0	22.5	33.9	3	53.2	0	161.5	1	1.6	16.27	0.470004
.	5	9.8	0	0	22.5	0	22.5	33.9	3	53.2	0	161.5	2	22.5	16.27	3.113515
.	5	20.7	0	0	1.5	0	14.6	32.4	3	53.2	0	161.5	1	1.5	16.27	0.405465
.	5	20.7	0	0	14.6	0	14.6	32.4	3	53.2	0	161.5	2	14.6	16.27	2.681022
.	5	23.5	4.7	250	0	1	0	32.8	3	53.2	0	161.5	1	0	16.27	0
.	5	33	0	0	1.7	0	15.9	32.8	3	53.2	0	161.5	1	1.7	16.27	0.530628
.	5	33	0	0	15.9	0	15.9	32.8	3	53.2	0	161.5	2	15.9	16.27	2.766319
.	5	35.2	2.4	125	0	1	0	32.6	3	53.2	0	161.5	1	0	16.27	0

.	5	45.1	0	0	1.8	0	16.6	33.1	3	53.2	0	161.5	1	1.8	16.27	0.587787
.	5	45.1	0	0	16.6	0	16.6	33.1	3	53.2	0	161.5	2	16.6	16.27	2.809403
.	5	46.1	2.4	125	0	1	0	33.1	3	53.2	0	161.5	1	0	16.27	0
.	5	58.5	2.4	125	0	1	0	32	3	53.2	0	161.5	1	0	16.27	0
.	5	69.4	0	0	1.8	0	16.7	32.9	3	53.2	0	161.5	1	1.8	16.27	0.587787
.	5	69.4	0	0	16.7	0	16.7	32.9	3	53.2	0	161.5	2	16.7	16.27	2.815409
.	5	71.1	2.4	125	0	1	0	33	3	53.2	0	161.5	1	0	16.27	0
.	5	83.8	2.4	125	0	1	0	32.8	3	53.2	0	161.5	1	0	16.27	0
.	5	95.3	2.4	125	0	1	0	37	3	53.2	0	161.5	1	0	16.27	0
.	5	104.8	0	0	1.8	0	13.7	37.8	3	53.2	0	161.5	1	1.8	16.27	0.587787
.	5	104.8	0	0	13.7	0	13.7	37.8	3	53.2	0	161.5	2	13.7	16.27	2.617396
.	5	107.5	2.4	125	0	1	0	37.7	3	53.2	0	161.5	1	0	16.27	0
.	5	120.9	0	0	2.2	0	18.8	37.4	3	53.2	0	161.5	1	2.2	16.27	0.788457
.	5	120.9	0	0	18.8	0	18.8	37.4	3	53.2	0	161.5	2	18.8	16.27	2.933857
.	4	0	8.3	500	0	1	0	36.1	1	60	0	80	1	0	17.12	0
.	4	1.9	0	0	11.6	0	11.6	34.1	1	60	0	80	2	11.6	17.12	2.451005
.	4	7.7	0	0	8.7	0	8.7	32.9	1	60	0	80	2	8.7	17.12	2.163323
.	4	12.4	5	300	0	1	0	33.1	1	60	0	80	1	0	17.12	0
.	4	20.4	0	0	12.8	0	12.8	32.7	1	60	0	80	2	12.8	17.12	2.549445
.	4	25.6	5	300	0	1	0	33.6	1	60	0	80	1	0	17.12	0
.	4	37.2	5	300	0	1	0	35.4	1	60	0	80	1	0	17.12	0
.	4	48.9	5	300	0	1	0	36.5	1	60	0	80	1	0	17.12	0
.	6	1.4	20	200	0	1	0	32	3	10	0	81	1	0	1.52	0
.	6	7.3	2.5	25	0	1	0	33.9	3	10	0	81	1	0	1.52	0
.	6	7.3	0	0	1.5	0	18.1	33.5	3	10	0	81	1	1.5	1.52	0.405465
.	6	7.3	0	0	18.1	0	18.1	33.5	3	10	0	81	2	18.1	1.52	2.895912
.	6	13.5	2.5	25	0	1	0	33.2	3	10	0	81	1	0	1.52	0
.	6	24	2.8	28	0	1	0	32.6	3	10	0	81	1	0	1.52	0
.	6	24.3	5	50	0	1	0	33.1	3	10	0	81	1	0	1.52	0
.	6	25.6	0	0	1.9	0	26.4	33.5	3	10	0	81	1	1.9	1.52	0.641854
.	6	25.6	0	0	26.4	0	26.4	33.5	3	10	0	81	2	26.4	1.52	3.273364
.	6	34.3	2.8	28	0	1	0	33.2	3	10	0	81	1	0	1.52	0
.	6	41.7	0	0	1.8	0	20.1	33.5	3	10	0	81	1	1.8	1.52	0.587787
.	6	41.7	0	0	20.1	0	20.1	33.5	3	10	0	81	2	20.1	1.52	3.00072

.	6	48	2.8	28	0	1	0	33.3	3	10	0	81	1	0	1.52	0
.	6	58.7	2.8	28	0	1	0	33.1	3	10	0	81	1	0	1.52	0
.	6	65.5	0	0	1.9	0	18.5	33.1	3	10	0	81	1	1.9	1.52	0.641854
.	6	65.5	0	0	18.5	0	18.5	33.1	3	10	0	81	2	18.5	1.52	2.917771
.	6	71	2.8	28	0	1	0	33	3	10	0	81	1	0	1.52	0
.	6	82.5	2.8	28	0	1	0	35.1	3	10	0	81	1	0	1.52	0
.	6	90	0	0	2	0	20.8	36.4	3	10	0	81	1	2	1.52	0.693147
.	6	90	0	0	20.8	0	20.8	36.4	3	10	0	81	2	20.8	1.52	3.034953
.	6	95.7	2.8	28	0	1	0	36.6	3	10	0	81	1	0	1.52	0
.	6	106	2.8	28	0	1	0	36.3	3	10	0	81	1	0	1.52	0
.	6	113	0	0	1.9	0	18.8	36.5	3	10	0	81	1	1.9	1.52	0.641854
.	6	113	0	0	18.8	0	18.8	36.5	3	10	0	81	2	18.8	1.52	2.933857
.	6	119	2.8	28	0	1	0	36.2	3	10	0	81	1	0	1.52	0
.	6	129.8	2.8	28	0	1	0	36.1	3	10	0	81	1	0	1.52	0
.	6	137.6	0	0	1.6	0	15.2	36.1	3	10	0	81	1	1.6	1.52	0.470004
.	6	137.6	0	0	15.2	0	15.2	36.1	3	10	0	81	2	15.2	1.52	2.721295
.	6	142.6	2.8	28	0	1	0	36.8	3	10	0	81	1	0	1.52	0
.	6	155.1	2.8	28	0	1	0	36.3	3	10	0	81	1	0	1.52	0
.	7	10	20	1200	0	1	0	37.4	3	60	0	158.8	1	0	13.32	0
.	7	21.1	3	180	0	1	0	34.8	3	60	0	158.8	1	0	13.32	0
.	7	30.7	3	180	0	1	0	34.5	3	60	0	158.8	1	0	13.32	0
.	7	42.3	3	180	0	1	0	34	3	60	0	158.8	1	0	13.32	0
.	7	54.6	3	180	0	1	0	34.1	3	60	0	158.8	1	0	13.32	0
.	7	66.6	3	180	0	1	0	33.5	3	60	0	158.8	1	0	13.32	0
.	7	78.7	3	180	0	1	0	33.7	3	60	0	158.8	1	0	13.32	0
.	7	81.9	0	0	2	0	18.9	33.3	3	60	0	158.8	1	2	13.32	0.693147
.	7	81.9	0	0	18.9	0	18.9	33.3	3	60	0	158.8	2	18.9	13.32	2.939162
.	7	89.8	3	180	0	1	0	33.8	3	60	0	158.8	1	0	13.32	0
.	9	71.7	10.6	35	0	1	0	36.1	1	3.3	1	52	1	0	0.04	0
.	9	73.8	2.4	8	0	1	0	35.5	1	3.3	1	52	1	0	0.04	0
.	9	74.5	0	0	0.7	0	6.5	35.4	1	3.3	1	52	1	0.7	0.04	-0.35667
.	9	74.5	0	0	6.5	0	6.5	35.4	1	3.3	1	52	2	6.5	0.04	1.871802
.	9	77.5	4.6	15	0	1	0	35.8	1	3.3	1	52	1	0	0.04	0
.	9	84	2.4	8	0	1	0	36.6	1	3.3	1	52	1	0	0.04	0

.	9	98	2.4	8	0	1	0	35.5	1	3.3	1	52	1	0	0.04	0
.	9	99.8	0	0	1.2	0	15.5	35.5	1	3.3	1	52	1	1.2	0.04	0.182322
.	9	99.8	0	0	15.5	0	15.5	35.5	1	3.3	1	52	2	15.5	0.04	2.74084
.	9	108.3	2.4	8	0	1	0	36.3	1	3.3	1	52	1	0	0.04	0
.	9	115	0	0	12.5	0	12.5	36.8	1	3.3	1	52	2	12.5	0.04	2.525729
.	9	120.2	2.4	8	0	1	0	36.5	1	3.3	1	52	1	0	0.04	0
.	9	132	2.4	8	0	1	0	36.4	1	3.3	1	52	1	0	0.04	0
.	9	143.5	2.4	8	0	1	0	36.7	1	3.3	1	52	1	0	0.04	0
.	9	156.9	2.4	8	0	1	0	36.1	1	3.3	1	52	1	0	0.04	0
.	10	3.9	17.7	460	0	1	0	32.9	1	26	1	112	1	0	5.74	0
.	10	11.9	0	0	2	0	17.1	32.7	1	26	1	112	1	2	5.74	0.693147
.	10	11.9	0	0	17.1	0	17.1	32.7	1	26	1	112	2	17.1	5.74	2.839078
.	10	15.1	3.9	100	0	1	0	32.2	1	26	1	112	1	0	5.74	0
.	10	27	3.9	100	0	1	0	33.4	1	26	1	112	1	0	5.74	0
.	10	38.9	3.9	100	0	1	0	34.9	1	26	1	112	1	0	5.74	0
.	12	30.2	20	112	0	1	0	31.4	1	5.6	0	61	1	0	0.33	0
.	12	33.2	0	0	2.4	0	17.7	32.1	1	5.6	0	61	1	2.4	0.33	0.875469
.	12	33.2	0	0	17.7	0	17.7	32.1	1	5.6	0	61	2	17.7	0.33	2.873565
.	12	42.8	3	16.8	0	1	0	33.7	1	5.6	0	61	1	0	0.33	0
.	12	53.6	3	16.8	0	1	0	35.2	1	5.6	0	61	1	0	0.33	0
.	12	55.3	0	0	1.9	0	22.6	35	1	5.6	0	61	1	1.9	0.33	0.641854
.	12	55.3	0	0	22.6	0	22.6	35	1	5.6	0	61	2	22.6	0.33	3.11795
.	12	66.4	3	16.8	0	1	0	34.8	1	5.6	0	61	1	0	0.33	0
.	12	72.3	0	0	1.7	0	16.6	34.1	1	5.6	0	61	1	1.7	0.33	0.530628
.	12	72.3	0	0	16.6	0	16.6	34.1	1	5.6	0	61	2	16.6	0.33	2.809403
.	12	78.1	3	16.8	0	1	0	35.2	1	5.6	0	61	1	0	0.33	0
.	12	90.3	3	16.8	0	1	0	35.9	1	5.6	0	61	1	0	0.33	0
.	12	96.4	0	0	1.7	0	15.7	36.5	1	5.6	0	61	1	1.7	0.33	0.530628
.	12	96.4	0	0	15.7	0	15.7	36.5	1	5.6	0	61	2	15.7	0.33	2.753661
.	12	102.9	3	16.8	0	1	0	35.5	1	5.6	0	61	1	0	0.33	0
.	12	113.9	3	16.8	0	1	0	37.2	1	5.6	0	61	1	0	0.33	0
.	12	120.3	0	0	1.8	0	16.9	36.2	1	5.6	0	61	1	1.8	0.33	0.587787
.	12	120.3	0	0	16.9	0	16.9	36.2	1	5.6	0	61	2	16.9	0.33	2.827314
.	12	126.2	3	16.8	0	1	0	35.9	1	5.6	0	61	1	0	0.33	0

.	12	139.1	3	16.8	0	1	0	35.2	1	5.6	0	61	1	0	0.33	0
.	12	143.9	0	0	1.4	0	16.3	36.1	1	5.6	0	61	1	1.4	0.33	0.336472
.	12	143.9	0	0	16.3	0	16.3	36.1	1	5.6	0	61	2	16.3	0.33	2.791165
.	12	151.3	3	16.8	0	1	0	36.8	1	5.6	0	61	1	0	0.33	0
.	12	162.1	0	0	1.3	0	15.5	36.3	1	5.6	0	61	1	1.3	0.33	0.262364
.	12	162.1	0	0	15.5	0	15.5	36.3	1	5.6	0	61	2	15.5	0.33	2.74084
.	12	163.5	3	16.8	0	1	0	36.3	1	5.6	0	61	1	0	0.33	0
.	13	1.7	20	100	0	1	0	34.3	1	5	0	58	1	0	0.22	0
.	13	12.8	2.5	12.5	0	1	0	32.8	1	5	0	58	1	0	0.22	0
.	13	15.1	0	0	1.4	0	13	32.3	1	5	0	58	1	1.4	0.22	0.336472
.	13	15.1	0	0	13	0	13	32.3	1	5	0	58	2	13	0.22	2.564949
.	13	19	10	50	0	1	0	32.9	1	5	0	58	1	0	0.22	0
.	13	32.4	2.5	12.5	0	1	0	34.4	1	5	0	58	1	0	0.22	0
.	13	37.9	0	0	1.6	0	12.1	35.2	1	5	0	58	1	1.6	0.22	0.470004
.	13	37.9	0	0	12.1	0	12.1	35.2	1	5	0	58	2	12.1	0.22	2.493205
.	13	43.4	2.5	12.5	0	1	0	36.7	1	5	0	58	1	0	0.22	0
.	13	55.6	2.5	12.5	0	1	0	38.3	1	5	0	58	1	0	0.22	0
.	13	69.5	2.5	12.5	0	1	0	36.9	1	5	0	58	1	0	0.22	0
.	13	75.1	0	0	1.1	0	11.1	38.2	1	5	0	58	1	1.1	0.22	0.09531
.	13	75.1	0	0	11.1	0	11.1	38.2	1	5	0	58	2	11.1	0.22	2.406945
.	13	79	2.5	12.5	0	1	0	37.6	1	5	0	58	1	0	0.22	0
.	13	92.3	2.5	12.5	0	1	0	37.6	1	5	0	58	1	0	0.22	0
.	13	98.6	0	0	0.7	0	6.7	37.7	1	5	0	58	1	0.7	0.22	-0.35667
.	13	98.6	0	0	6.7	0	6.7	37.7	1	5	0	58	2	6.7	0.22	1.902108
.	13	101.4	5	25	0	1	0	37.3	1	5	0	58	1	0	0.22	0
.	13	103.8	2.5	12.5	0	1	0	37	1	5	0	58	1	0	0.22	0
.	13	110.9	0	0	0.5	0	4.8	37.1	1	5	0	58	1	0.5	0.22	-0.69315
.	13	110.9	0	0	4.8	0	4.8	37.1	1	5	0	58	2	4.8	0.22	1.568616
.	13	113.6	5	25	0	1	0	36.9	1	5	0	58	1	0	0.22	0
.	13	115.7	2.5	12.5	0	1	0	36.6	1	5	0	58	1	0	0.22	0
.	13	126.8	3	15	0	1	0	37.5	1	5	0	58	1	0	0.22	0
.	13	134.7	0	0	0.3	0	2.8	37.7	1	5	0	58	1	0.3	0.22	-1.20397
.	13	134.7	0	0	2.8	0	2.8	37.7	1	5	0	58	2	2.8	0.22	1.029619
.	13	140.3	6	30	0	1	0	37.4	1	5	0	58	1	0	0.22	0

.	19	0.5	9.3	120	0	1	0	34.4	1	12.9	1	82.6	1	0	0.1	0
.	19	11	1.4	18	0	1	0	33.8	1	12.9	1	82.6	1	0	0.1	0
.	19	23	1.4	18	0	1	0	33.2	1	12.9	1	82.6	1	0	0.1	0
.	19	33	0	0	1.3	0	7.2	34.3	1	12.9	1	82.6	1	1.3	0.1	0.262364
.	19	33	0	0	7.2	0	7.2	34.3	1	12.9	1	82.6	2	7.2	0.1	1.974081
.	19	35	1.4	18	0	1	0	34.6	1	12.9	1	82.6	1	0	0.1	0
.	19	41	1.4	18	0	1	0	36.2	1	12.9	1	82.6	1	0	0.1	0
.	19	47	1.5	19.8	0	1	0	36.6	1	12.9	1	82.6	1	0	0.1	0
.	19	58	0	0	1.2	0	7.1	37.4	1	12.9	1	82.6	1	1.2	0.1	0.182322
.	19	58	0	0	7.1	0	7.1	37.4	1	12.9	1	82.6	2	7.1	0.1	1.960095
.	19	59	1.5	19.8	0	1	0	37.8	1	12.9	1	82.6	1	0	0.1	0
.	19	61	4.7	60	0	1	0	37.4	1	12.9	1	82.6	1	0	0.1	0
.	19	69	0	0	2.2	0	13	37.8	1	12.9	1	82.6	1	2.2	0.1	0.788457
.	19	69	0	0	13	0	13	37.8	1	12.9	1	82.6	2	13	0.1	2.564949
.	19	74	1.9	25	0	1	0	36.6	1	12.9	1	82.6	1	0	0.1	0
.	19	81	0	0	2.1	0	13	37.2	1	12.9	1	82.6	1	2.1	0.1	0.741937
.	19	81	0	0	13	0	13	37.2	1	12.9	1	82.6	2	13	0.1	2.564949
.	19	86	1.9	25	0	1	0	36.2	1	12.9	1	82.6	1	0	0.1	0
.	19	93	0	0	1.5	0	8.7	35.6	1	12.9	1	82.6	1	1.5	0.1	0.405465
.	19	93	0	0	8.7	0	8.7	35.6	1	12.9	1	82.6	2	8.7	0.1	2.163323
.	19	95	2.3	30	0	1	0	35	1	12.9	1	82.6	1	0	0.1	0
.	19	99	2.3	30	0	1	0	35.6	1	12.9	1	82.6	1	0	0.1	0
C	19	99	0	0	5.1	0	54.9	35.6	1	12.9	1	82.6	1	5.1	0.1	1.629241
C	19	99	0	0	54.9	0	54.9	35.6	1	12.9	1	82.6	2	54.9	0.1	4.005513
.	19	105	0	0	2	0	12.7	36.4	1	12.9	1	82.6	1	2	0.1	0.693147
.	19	105	0	0	12.7	0	12.7	36.4	1	12.9	1	82.6	2	12.7	0.1	2.541602
.	19	111	2.3	30	0	1	0	36.4	1	12.9	1	82.6	1	0	0.1	0
.	19	117	0	0	1.9	0	11.8	36.8	1	12.9	1	82.6	1	1.9	0.1	0.641854
.	19	117	0	0	11.8	0	11.8	36.8	1	12.9	1	82.6	2	11.8	0.1	2.4681
.	19	123	2.3	30	0	1	0	36.6	1	12.9	1	82.6	1	0	0.1	0
.	19	128	0	0	2	0	12.3	36.8	1	12.9	1	82.6	1	2	0.1	0.693147
.	19	128	0	0	12.3	0	12.3	36.8	1	12.9	1	82.6	2	12.3	0.1	2.509599
.	19	135	2.3	30	0	1	0	36.2	1	12.9	1	82.6	1	0	0.1	0
.	19	141	0	0	1.4	0	9.1	36.2	1	12.9	1	82.6	1	1.4	0.1	0.336472

.	19	141	0	0	9.1	0	9.1	36.2	1	12.9	1	82.6	2	9.1	0.1	2.208274
.	19	147	2.3	30	0	1	0	35.8	1	12.9	1	82.6	1	0	0.1	0
.	19	152	0	0	1.3	0	9.9	35.2	1	12.9	1	82.6	1	1.3	0.1	0.262364
.	19	152	0	0	9.9	0	9.9	35.2	1	12.9	1	82.6	2	9.9	0.1	2.292535
.	19	159	2.3	30	0	1	0	35.2	1	12.9	1	82.6	1	0	0.1	0
.	19	166	0	0	1.3	0	8.9	35.2	1	12.9	1	82.6	1	1.3	0.1	0.262364
.	19	166	0	0	8.9	0	8.9	35.2	1	12.9	1	82.6	2	8.9	0.1	2.186051
.	19	171	0	0	1.1	0	7.6	35.6	1	12.9	1	82.6	1	1.1	0.1	0.09531
.	19	171	0	0	7.6	0	7.6	35.6	1	12.9	1	82.6	2	7.6	0.1	2.028148
.	19	172	2.3	30	0	1	0	35.8	1	12.9	1	82.6	1	0	0.1	0
.	21	103	20	1000	0	1	0	36.8	1	50	1	172	1	0	17.2	0
.	21	111	0	0	2.1	0	20.6	38.7	1	50	1	172	1	2.1	17.2	0.741937
.	21	111	0	0	20.6	0	20.6	38.7	1	50	1	172	2	20.6	17.2	3.025291
.	21	116	5	250	0	1	0	37.6	1	50	1	172	1	0	17.2	0
.	21	127	0	0	2.2	0	25.2	38	1	50	1	172	1	2.2	17.2	0.788457
.	21	127	0	0	25.2	0	25.2	38	1	50	1	172	2	25.2	17.2	3.226844
.	21	128	5	250	0	1	0	38	1	50	1	172	1	0	17.2	0
.	21	139	2.5	125	0	1	0	38.6	1	50	1	172	1	0	17.2	0
.	21	150	2.5	125	0	1	0	37.2	1	50	1	172	1	0	17.2	0
.	21	159	0	0	3.4	0	27.2	37.6	1	50	1	172	1	3.4	17.2	1.223775
.	21	159	0	0	27.2	0	27.2	37.6	1	50	1	172	2	27.2	17.2	3.303217
.	21	167	0	0	2.8	0	25.8	37.8	1	50	1	172	1	2.8	17.2	1.029619
.	21	167	0	0	25.8	0	25.8	37.8	1	50	1	172	2	25.8	17.2	3.250374
.	32	9.4	0	0	1.8	0	15.3	32.3	2	10	0	60.5	1	1.8	0.98	0.587787
.	32	9.4	0	0	15.3	0	15.3	32.3	2	10	0	60.5	2	15.3	0.98	2.727853
.	32	13.4	2.5	25	0	1	0	33.2	2	10	0	60.5	1	0	0.98	0
.	32	25.1	2.5	25	0	1	0	30.8	2	10	0	60.5	1	0	0.98	0
.	32	33.1	0	0	1.9	0	12.3	30.1	2	10	0	60.5	1	1.9	0.98	0.641854
.	32	33.1	0	0	12.3	0	12.3	30.1	2	10	0	60.5	2	12.3	0.98	2.509599
.	32	37.3	2.5	25	0	1	0	30.1	2	10	0	60.5	1	0	0.98	0
.	32	49.1	2.5	25	0	1	0	31.5	2	10	0	60.5	1	0	0.98	0
.	32	55.4	0	0	2.2	0	17.8	31.2	2	10	0	60.5	1	2.2	0.98	0.788457
.	32	55.4	0	0	17.8	0	17.8	31.2	2	10	0	60.5	2	17.8	0.98	2.879198
.	32	61.6	2.5	25	0	1	0	32.2	2	10	0	60.5	1	0	0.98	0

.	32	61.5	2.5	25	0	1	0	33.3	2	10	0	60.5	1	0	0.98	0
.	32	80.9	0	0	2.5	0	15.9	34.9	2	10	0	60.5	1	2.5	0.98	0.916291
.	32	80.9	0	0	15.9	0	15.9	34.9	2	10	0	60.5	2	15.9	0.98	2.766319
.	32	87.4	1.8	18	0	1	0	36.7	2	10	0	60.5	1	0	0.98	0
.	32	92.8	0	0	3	0	18.6	37.2	2	10	0	60.5	1	3	0.98	1.098612
.	32	92.8	0	0	18.6	0	18.6	37.2	2	10	0	60.5	2	18.6	0.98	2.923162
.	33	102	20	134	0	1	0	37	3	6.7	1	61	1	0	0.29	0
.	33	115	2.5	17	0	1	0	36.8	3	6.7	1	61	1	0	0.29	0
.	33	115	0	0	1.7	0	9.3	36.8	3	6.7	1	61	1	1.7	0.29	0.530628
.	33	115	0	0	9.3	0	9.3	36.8	3	6.7	1	61	2	9.3	0.29	2.230014
.	33	117	0	0	1.7	0	9.5	36.8	3	6.7	1	61	1	1.7	0.29	0.530628
.	33	117	0	0	9.5	0	9.5	36.8	3	6.7	1	61	2	9.5	0.29	2.251292
.	33	127.4	2.5	17	0	1	0	36.2	3	6.7	1	61	1	0	0.29	0
.	33	139	2.5	17	0	1	0	37	3	6.7	1	61	1	0	0.29	0
.	33	139	0	0	0.9	.	5	37	3	6.7	1	61	1	0.9	0.29	-0.10536
.	33	139	0	0	5	.	5	37	3	6.7	1	61	2	5	0.29	1.609438
.	33	148	10.5	70	0	1	0	36.8	3	6.7	1	61	1	0	0.29	0
.	33	150.7	2.8	19	0	1	0	36.4	3	6.7	1	61	1	0	0.29	0
.	34	4.7	20.1	880	0	1	0	34.5	3	43.8	0	156	1	0	17.64	0
.	34	7	0	0	2.8	0	24.1	35.4	3	43.8	0	156	1	2.8	17.64	1.029619
.	34	7	0	0	24.1	0	24.1	35.4	3	43.8	0	156	2	24.1	17.64	3.182212
.	34	17.2	0	0	2	0	20	33.6	3	43.8	0	156	1	2	17.64	0.693147
.	34	17.2	0	0	20	0	20	33.6	3	43.8	0	156	2	20	17.64	2.995732
C	35	-0.5	32	140	0	1	0	31.6	1	4.38	1	65	1	0	0.53	0
C	35	1	0	0	28.8	0	28.8	31.6	1	4.38	1	65	2	28.8	0.53	3.360375
.	35	19	0	0	1.5	0	5.8	32.8	1	4.38	1	65	1	1.5	0.53	0.405465
.	35	19	0	0	5.8	0	5.8	32.8	1	4.38	1	65	2	5.8	0.53	1.757858
.	35	43	0	0	1.2	0	6.2	36.2	1	4.38	1	65	1	1.2	0.53	0.182322
.	35	43	0	0	6.2	0	6.2	36.2	1	4.38	1	65	2	6.2	0.53	1.824549

B.2. VARIABLES AND DESCRIPTIONS FOR NONMEM INPUT DATA FILE

The following table lists the variables included in the NONMEM input data file and a description of what each variable is and how it is recorded.

Variable Name	Variable Description	Excel Dataset or View/ Reference Variable	Additional Information / Default Values	Required Format / Examples
C	Omit Column	Dosing records are commented out (C ='C') which are after last PK collection.	Default value is C for commented out records.	
SUBJID	Protocol specified subject number	All datasets / USUBJID	Exclude '-' from SUBJID and convert to numeric value.	integer up to 4 digits NUM: 4.
NID	New Sequential number ID	Derived beginning at 1 across combined protocols	Unique for each subject, incrementing by 1 for each new subject Default: Initial value = 1.	integer up to 2 digits NUM: 2.
AMT	The actual amount of dose received		Only populate for dosing records. Set to missing '.' for concentration records. Units = mg	
AMT (weight corrected)		Derived from AMT/Weight	Units = mg/kg	

Variable Name	Variable Description	Excel Dataset or View/ Reference Variable	Additional Information / Default Values	Required Format / Examples
TACA	Time After Cardiac Arrest	Derived from time following the cardiac arrest	Units = Hours	NUM: 5.
TAFD	Time After Subject's Very First Treatment Dose	Derived from first dosing date and time	Units = Hours	
TAD	Time After Dose		Units = Hours	NUM: 5.
EVID	Event ID	Derived	0=observation, 1=dose	NUM: 1.
DV	Dependent variable column	PC/PCSTRESC Set to '.' for Dose records.	Units: IU/dL	NUM: BEST8.
LDV	Log-transformed DV	If DV ne '.'and DV>0 then LDV=log(DV)	Set to missing '.' for Dosing records.	NUM: BEST8.
MDV	Missing Data Value	0 = observation; 1 = dose or missing observation		NUM: 5.
FREE	Free phenytoin level		Units = µg/mL	
WT	Weight of subject		Units = kg	NUM: BEST5.
HT	Height of subject		Units = cm	
TEMP	Temperature of subject		Units = Celsius	

Variable Name	Variable Description	Excel Dataset or View/ Reference Variable	Additional Information / Default Values	Required Format / Examples
FENT FLAG	Fentanyl Administration Flag		1: Fentanyl administered records 0: None	NUM: 5.
LEV FLAG	LEV Administration Flag		1: LEV administered records 0: None	
ECMO FLAG	ECMO flag		1: Subject with ECMO 0: No ECMO	
TRT	Actual Treatment Code		1: Phenytoin 2: Levetiracetam 3: Both	NUM: 5.
ARM	Arm Code	DM/ARM	Cooling Duration: 1: 24 hours 2: 48 hours 3: 72 hours 4: Came in Cold 5: No cooling	NUM: 5.
SEX	Sex	DM / SEX	0: Female 1: Male Set missing or 'U' to '.'	NUM: 5.
AGE	Age of subject	DM / AGE	Units = years	NUM: 5.
CPR MINS	Length of cardiac arrest		Units = mins	NUM: 5.
ALBH	Albumin levels		Units = µg/dL	
BUNH	Blood urea nitrogen		Units = mg/dL	

Variable Name	Variable Description	Excel Dataset or View/ Reference Variable	Additional Information / Default Values	Required Format / Examples
CRH	Creatinine		Units = mg/dL	
BILIH	Bilirubin		Units = mg/dL	
ALTH	Alanine Aminotransferase		Units = IU/L	
ASTH	Aspartate Aminotransferase		Units = IU/L	

B.3. BOOTSTRAP ANALYSIS AND SIMULATION OF PHENYTOIN PK PARAMETERS

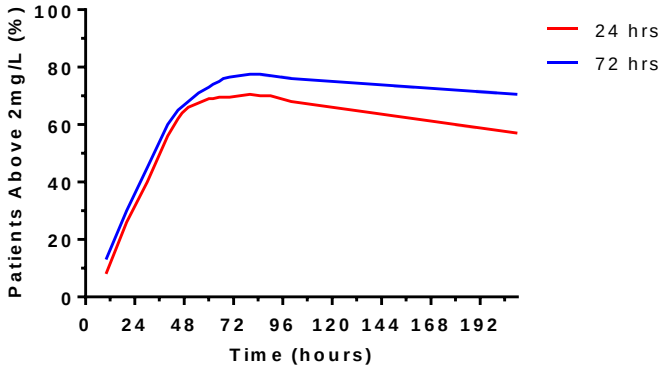
A bootstrap analysis was used to evaluate the final model and the robustness of final parameters. The precision of the final parameters were determined from 500 bootstrap data analysis using PsN and Pirana. Values are expressed as 5^{th} - 95^{th} percentile. Bootstrap estimates were in good agreement with final parameter estimates.

Parameter	Estimate (Median and 90% CI)	ISV (Inter-Subject Variability)
V_1 (L)	330 (305 - 386)	7.24 (3.12 - 12.4)
V_{max0} (mg/h)	6.30 (4.23 - 8.79)	48.7 (21.0 – 89.3)
V_{maxi} (mg/h)	13.5 (1.35 - 92.7)	96.2 (5.9 – 157.4)
k_m (mg/L)	3.07 (0.247 – 3.875)	-
k_{ind} (h^{-1})	0.005 (0.0004 - 0.0161)	-

Footnote: ISV = inter-subject variability expressed as coefficient of variation (%);V_1 = apparent volume of distribution; V_{max0} = time-invariant maximum velocity of metabolism at baseline; V_{maxi} = time-dependent velocity defined by the rate constant kind and time t; k_m = Michaelis-Menten elimination rate constant; k_{ind} = rate constant for induction of metabolism.

Using results of the final PopPK model of phenytoin, simulations were performed to determine phenytoin elimination under clinically relevant conditions. A 1000 pediatrics receiving a standard loading dose of fosphenytoin (20mg/kg) followed by maintenance doses and cooled to

33°C for 24 or 72 hours was simulated. A population median and 90% confidence interval was determined. Cooling for 24 or 72 hours demonstrated a decrease in phenytoin elimination, which led to elevated phenytoin concentrations (above the therapeutic range of 2 mg/L).



Simulated phenytoin concentrations from 1000 pediatric subjects receiving a standard loading dose of fosphenytoin (20mg/kg) following by maintenance doses and receiving therapeutic hypothermia to 24 or 72 hours.

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