

Cardiac arrest and therapeutic hypothermia interaction with isoform-specific cytochrome P450 drug metabolism

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d) A list of nonstandard abbreviations used in the paper:

Cardiac arrest (CA)

Systemic clearance (CL)

Volume of distribution –central compartment (V_1)

Volume of distribution –peripheral compartment (V_2)

Inter-compartment clearance (Q)

Area under curve (AUC)

Nonlinear mixed effect modeling program (NONMEM)

Pharmacokinetics (PK)

Pharmacodynamics (PD)

Mean arterial pressure (MAP)

Blood urea nitrogen (BUN)

Objective function value (OFV)

Population predicted concentrations (PRED)

Observed concentrations (OBS)

Individual predicted concentrations (IPRED)

Weighted residuals (WRES)

Ultra performance liquid chromatography- triple quadrupole mass spectrometry (UPLC/MS/MS)

Electrospray ionization (ESI)

Intensive care unit (ICU)

ABSTRACT

Mild therapeutic hypothermia is emerging clinically as a neuroprotection therapy for cardiac arrest (CA) victims, however its effects combined with the pathogenesis of critical illness on drug disposition and response have not been fully elucidated. We determined the activities of four major hepatic metabolizing enzymes (CYP3A, CYP2C, CYP2D and CYP2E) during hypothermia after experimental CA in rats by evaluating the pharmacokinetics (PK) of their probe drugs as a function of altered body temperature. Animals were randomized into sham normothermia (37.5-38°C), CA normothermia, sham hypothermia (32.5-33°C), and CA hypothermia groups. Probe drugs (midazolam, diclofenac, dextromethorphan and chlorzoxazone) were given simultaneously by IV bolus after temperature stabilization. Multiple blood samples were collected between 0-8 hours after drug administration. PK analysis was conducted using a non-compartmental approach and population PK modelling. Non-compartmental analysis showed that the clearance of midazolam (CYP3A) in CA hypothermia was reduced from sham normothermia (681.6±190.0 mL/hr/kg vs 1268.8±348.9 mL/hr/kg, $p<0.05$). The clearance of chlorzoxazone (CYP2E) in CA hypothermia was reduced from sham normothermia (229.6±75.6 mL/hr/kg vs 561.89±215.9 mL/hr/kg, $p<0.05$). Population PK analysis further demonstrated the decreased clearance of midazolam (CYP3A) was associated with CA injury ($p<0.05$). The decreased clearance of chlorzoxazone (CYP2E) was also associated with CA injury ($p<0.01$). Hypothermia was found associated with the decreased volume of distribution of midazolam (V_1), dextromethorphan (V_1) and peripheral compartment for chlorzoxazone (V_2) ($p<0.05$, $p<0.05$, and $p<0.01$, respectively). Our data indicated that hypothermia, CA and interaction lead to CYP isoform specific alteration in activities and magnitudes in drug metabolism and disposition.

INTRODUCTION

Brain injury is a common cause of morbidity and mortality after resuscitation from cardiac arrest (CA) (Geocadin et al., 2008, Manole et al., 2009). Therapeutically reducing body temperature to 32-35°C over 12 to 24 hours after out-of-hospital CA is the proven neuroprotective strategy in these highly susceptible patients (Bernard et al., 2002; Hypothermia after cardiac arrest study group, 2002; Holzer, 2010). Despite the benefit of therapeutic hypothermia, whole body cooling after resuscitation complicates drug therapy due to its potential effects on both drug disposition and response. Previous studies have found significant elevated drug levels and prolonged drug response during hypothermia for drugs such as phenytoin, vecuronium, morphine, propofol, and fentanyl (Iida et al., 2001; Caldwell et al., 2000; Roka et al., 2008; Leslie et al., 1995; Fritz et al., 2005). Given the large number of medications used in critically ill patients after CA and possible adverse drug effects, a close pharmacokinetic (PK) and pharmacodynamic (PD) monitoring is required by critical care practitioners (Cullen et al., 1997; Lazarou et al., 1998; Vargas et al., 1998). Currently, the specific changes in drug metabolism and disposition during hypothermia after CA are largely unknown.

Many of the drugs used in CA patients do not have readily measurable pharmacodynamic (PD) endpoints and have narrow therapeutic indices. This fact necessitates the evaluation of a systemic drug metabolism and PK as a tool to ensure drug safety and efficacy. Cytochrome P450 (CYP) enzymes play an essential role in metabolizing medications commonly used in critical care medicine (Tortorici et al., 2007), including benzodiazepines, calcium channel blockers, anesthetics, and opioids. The magnitude of altered activity of specific CYP isoforms in CA with changes in body temperature is poorly understood. In addition, during hypothermia post CA, the

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

In this study, we estimated the activities of four major CYP isoforms CYP3A, CYP2C, CYP2D and CYP2E. A probe cocktail of midazolam, diclofenac, dextromethorphan, and chlorzoxazone was utilized for the phenotypic assessment of CYP3A, CYP2C, CYP2D, and CYP2E1 activity, respectively. In vivo validation of all these probe drugs or in combination in cocktail has been previously tested (Scott et al., 1999; Xia, et al., 2010; Yu et al., 2007; Ghassabian et al., 2009; Krösser et al., 2006; Blakey et al., 2004). Midazolam, diclofenac, dextromethorphan and chlorzoxazone were also chosen based on their relative specificity, their relatively short half-life, and their prior clinical use as probe drugs (Frye 2004; Streetman et al., 2000). In addition, these drugs were selected based on the fact that mass spectrometric detection limits allowed for lower doses of these drugs in rats to decrease the likelihood of probe drug interactions upon co-administration. We hypothesized that the metabolisms of CYP probe substrates decrease during cooling due to the acute effect of hypothermia on the enzyme activity and that these alterations would be isoform specific. The first endpoint of the study is to evaluate systemic clearance of probe drugs within different groups by noncompartmental PK analysis. The secondary endpoint is to determine the specific correlation between hypothermia and CA with probe drug pharmacokinetics by nonlinear mixed effect PK modeling.

METHODS

Animals

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

Surgery and cardiac arrest procedure

The animals were randomly divided into four groups: (a) sham normothermia, (b) sham hypothermia, (c) CA normothermia, and (d) CA hypothermia (n=6). Rats were anesthetized with 1-1.5% isoflurane via a nose cone, tracheally intubated, and mechanically ventilated to maintain eucapnia (PaCO₂ between 35 and 45 mm Hg) to start surgery and cannulation. Anesthesia was maintained throughout the experiment with isoflurane and oxygen. The left femoral artery was cannulated for measurement of mean arterial pressure, and the femoral vein was cannulated bilaterally for drug administration and blood sampling. Neuromuscular blockade was induced and maintained with vecuronium 2 mg/kg intravenously before the cannulation. After preparative surgery, isoflurane was washed out with 100% oxygen for 3 minutes and room air for 2 minutes. CA was initiated by asphyxiation via disconnection of the mechanical ventilation. Asphyxial CA was continued for 8 minutes, with approximately 6 minutes of asystole, confirmed by electrocardiogram. Resuscitation was initiated by reconnecting the rat to the ventilator, administering intravenous epinephrine (0.005 mg/kg) and sodium bicarbonate (1mEq/kg), and performing manual chest compressions for about 40-60 seconds until restoration of spontaneous

circulation. Sham animals received all of the above surgeries for blood sample collection but were not made hypothermic or subjected to cardiac arrest.

Hypothermia and temperature measurement

Hypothermia was induced starting 60 minutes after resuscitation. Systemic hypothermia with a target temperature of 32.5-33°C was initiated by surface cooling for 30 minutes, followed by 15 minutes stabilization. The animals were maintained in a hypothermic state for 8-hr after temperature stabilization. Rectal temperature of the normothermic group was maintained at 37.5-38°C. Body temperature was continuously measured using a rectal temperature probe. A heating pad, heating light, cooling fan and ice bags were used to adjust and maintain the target temperature.

Drug administration and pharmacokinetics sampling

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

Arterial blood gas sampling

Multiple arterial blood gas samples (100 μ L) were collected (at baseline before CA, 10, 30, 60 minutes after return of spontaneous circulation, at the end of hypothermia induction, and every hour during the blood sampling) for the purpose of monitoring the status of the animals over the time. Physiological parameters including pH, MAP, PaCO₂, pH, lactate, hematocrit, K⁺, Mg²⁺, glucose, and blood urea nitrogen (BUN) were measured at each time point. A maintenance infusion of saline (3mL/hr/kg) was started beginning at 20 minutes after drug administration and continued for 8 hours. Doses of bicarbonate were given as needed to correct acidosis.

Bioanalytical analysis by UPLC/MS/MS

Probe drug plasma concentrations were measured simultaneously by ultra performance liquid chromatography- triple quadrupole mass spectrometry (UPLC/MS/MS). Waters UPLC system with ACQUITY BEH C18 1.7 μ m \times 2.1mm \times 50mm UPLC column was used. The mobile phase consisted of 0.1% formic acid in water (A) and pure acetonitrile (B), gradient from 90:10 (A:B) to 70:30 (A:B) within 3 minutes and return to 90:10 (A:B) at 5.0 minutes at a flow rate of 0.25mL/min. Thermo triple-quadrupole mass spectrometer, equipped with electrospray ionization (ESI) source was used for mass analysis and detection (Quantum Ultra, Thermo Fisher Scientific, San Jose, CA). All plasma samples were extracted by solid phase extraction using Waters 1cc Oasis HLB cartridges (Waters, Milford, MA, USA) with manufacture standard protocol. The specific ion transition for midazolam, diclofenac, dextromethorphan and chlorzoxazone were 325.6>291, 294>250, 272>171, 168.6>132, respectively. The assay has been validated with the linearity range from 0.2-250ng/mL for midazolam and 2.5-500ng/mL for diclofenac, dextromethorphan and chlorzoxazone. Assay accuracy (three days) is greater than

86.4% for all four probe drugs. Inter-day and intra-day variability were < 20.0% and <18.1%, respectively. The Xcalibur 2.0 software was used for data acquisition and analysis.

Non-compartment al model analysis

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

Population pharmacokinetic modelling

The population pharmacokinetic model was constructed using a nonlinear mixed-effects approach as implemented in the NONMEM VI program (Icon, Hanover, Maryland). The first order conditional estimation method (FOCE) with interaction was used to estimate all parameters. The population pharmacokinetic model consisted of a pharmacokinetic structural model and a statistical model in which between subject and within subject variability were described. One and two-compartment structural pharmacokinetics models were investigated as base model structures. The one-compartment model structure was evaluated using the ADVAN 1 TRANS 2 subroutine (estimate CL and V) and the two-compartment model structure was implemented as the ADVAN3 TRANS4 subroutine (estimate CL, V1, V2 and Q). The inter-individual variability in the pharmacokinetic parameters was assumed to be log-normally distributed for all population parameters. Inter-individual variability in model parameters will be modeled using an exponential term. $\theta_i = \theta \times e^{\eta_i}$ with $\eta_i \sim N(0, \omega^2)$, θ is the population value and η_i is the variable accounting for inter-individual variability with mean zero and variance ω^2 . Residual

variability (σ^2), the discrepancy between the individual observed ($C_{obs,ij}$) and the individual model-predicted ($C_{pred,ij}$) plasma concentrations, was evaluated using additive, proportional or a combined additive and proportional model $C_{obs,ij} = C_{pred,ij} \times (1 + \epsilon_{ij1} + \epsilon_{ij2})$, Where $C_{obs,ij}$ is the j th measured observation in individual i , $C_{pred,ij}$ is the j th model predicted value in individual i , ϵ_{ij} is the residual error. Further refinement of the error model was performed as needed. The model building process was guided by analyzing the goodness of fit plots, plausibility of parameter estimates, precision of parameters estimates, and the lowest objective function value (OFV) provided by NONMEM. Final model parameters were reported with estimates mean with standard error (se%).

Covariate model

After a base model was chosen for each drug, covariate effects on the parameters of that model were evaluated in the following way. Factors treated as categorical covariates were temperature (TEMG, 0= normothermia, 1=hypothermia), and CA injury (CAG, 0= sham, 1=CA). They have been tested in forward addition in either of the following ways: $P_{TV} = \theta_1 + \theta_2 \times (Cov)$ or $P_{TV} = \theta_1 \times \theta_2^{(Cov)}$, where θ_1 and θ_2 are estimated fixed effect parameters and Cov is the subject specific value of the categorical covariate. Three physiological parameters (Glucose, BUN and K) were tested in forward fashion as continuous covariates, which has been tested in the following: $P_{TV} = \theta_1 \times \theta_2^{(Cov/Medcov)}$, where θ_1 and θ_2 are estimated fixed effect parameters, Cov is the subject specific value of the continuous covariate, and MedCov is the median value of the Cov. To assess whether the model with covariates statistically improved the fit to the data from base model, the difference between their objective function values, referred to as the log likelihood ratio, was calculated. This ratio was assumed to be chi-square (χ^2) distributed. A decrease in the OFV of 3.84 and 6.63 is considered to be significant at $p=0.05$ and $p=0.01$ respectively, with 1

degree of freedom. For the final model, stepwise forward addition and then backward elimination process was used.

The final covariate models were also evaluated using goodness of fit diagnostic plots, successful minimization, precision for all parameters (se %) and a visual predictive check. For a predictive check, 1000 Monte Carlo simulation replicates of the original data set were generated using the final population model estimates. These simulated curves were compared to the observed data. The Empirical Bayes Estimates from NONMEM represented the parameters for individual subjects for each time point. The individual clearances generated for each time point from each of the four groups were visualized as a graph for further evaluation.

Other Statistical analysis

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

RESULTS

Temperature curves and physiological parameters

The body temperatures at sham normothermia, sham hypothermia, CA normothermia, and CA hypothermic groups were $37.9\pm 0.4^{\circ}\text{C}$, $32.5\pm 0.4^{\circ}\text{C}$, $37.8\pm 0.5^{\circ}\text{C}$, and $32.8\pm 0.3^{\circ}\text{C}$, respectively.

Time temperature curves of four treatment groups have been depicted in Figure 1.

Physiological parameters of MAP, PaCO₂, pH, glucose, lactate, BUN, hematocrit, Mg²⁺ and K⁺ from each treatment group at each time point (baseline, 1-hr after resuscitation, end of cooling induction, 4-hr cooling, 8-hr cooling) are shown in Table 1. At 1-hr after resuscitation, MAP in both CA groups was lower than in the sham control groups, and then they recovered with the time. PaCO₂ level was lower in CA groups than sham groups at 1-hr after resuscitation. At 1-hr after resuscitation, the glucose level in the CA hypothermia was the lowest among groups. BUN increased over the time in all four groups, especially in CA groups. pH in the CA hypothermia group decreased from the end of cooling induction compared to baseline. Mg²⁺ and K⁺ levels in CA groups both increased over the time.

Time concentration curves and systemic clearance from non-compartment model analysis

Time-concentration curves from four groups for midazolam, diclofenac, dextromethorphan and chlorzoxazone were shown in Figure 2. Based on time concentration profile, systemic clearances calculated by non-compartmental analysis were shown in Figure 3. The clearances of midazolam in sham hypothermia, CA normothermia and CA hypothermia groups were 83.2%, 62.4% and 52.5% of those in the sham normothermia group, respectively. Significant reduction in the clearance of midazolam in CA hypothermia was found compared with the sham normothermic

control (681.6 ± 190.0 mL/hr/kg vs 1268.8 ± 348.9 mL/hr/kg, $p < 0.05$). The clearances of diclofenac in sham hypothermia, CA normothermia and CA hypothermia groups were 89.6%, 102.8% and 75.5% compared to those in sham normothermia group. There was no significant difference found in clearances of diclofenac among four groups. The clearances of dextromethorphan in sham hypothermia, CA normothermia and CA hypothermia groups were 106.9%, 111.5% and 79.9% of those in the sham normothermia group, respectively. There was no significant difference found in clearances of dextromethorphan among four groups. The clearances of chlorzoxazone in sham hypothermia, CA normothermia and CA hypothermia groups were 63.1%, 45.3% and 40.9% of the sham normothermia group, respectively. Significant reductions in clearances of chlorzoxazone for CA normothermia and CA hypothermia were observed when compared to those in sham normothermia (254.7 ± 55.5 mL/hr/kg, 229.6 ± 75.6 mL/hr/kg, vs 561.8 ± 215.9 mL/hr/kg, $p < 0.05$).

Population pharmacokinetic modeling analysis

The two-compartment model structure was significantly better than the one compartment model for the disposition of midazolam, diclofenac, dextromethorphan and chlorzoxazone (OFV difference at least 16.0 points, 2df, $p < 0.001$). Inter-individual variability (ω^2) terms were needed for CL, V1, Q, V2 in models for midazolam, diclofenac, and dextromethorphan and for CL, Q and V2 in model for chlorzoxazone. The residual error model used for all four models was $C_{obs,ij} = C_{pred,ij} \times (1 + \epsilon_{ij1})$. Each significant covariate in the final model has lowered OFV value greater than 3.84 ($p < 0.05$) than the OFV value of the base model. Parameter estimates and standard errors (se%) calculated from the covariance step in NONMEM were shown in Table 2. Goodness-of-fit diagnostic plots, including individual predicted concentration-observed

concentration (IPRED-OBS) and weighted residuals-time (WRES-TIME) of the final models for midazolam, diclofenac, dextromethorphan and chlorzoxazone were depicted in Figure 4.

The final pharmacokinetic model for midazolam included a CA categorical (CAG) covariate in systemic clearance (CL), and a temperature categorical (TEMG) covariate in central volume of distribution (V_1). CA groups showed the decreased clearance of midazolam, which was described in the equation: $CL(L/hr/kg) = 0.990 + CAG \times (-0.318)$. The systemic clearance of midazolam in CA groups was estimated to be 67.9% of those in sham groups. Hypothermia was associated with a decrease in the V_1 of midazolam. The specific relationship has been shown in the equation: $V_1(L/hr) = 0.258 + TEMG \times (-0.0786)$. The V_1 of midazolam in the hypothermic groups was estimated to be 69.5% of that seen in normothermia. The final pharmacokinetic model for diclofenac included a temperature (TEMG) covariate in CL, which can be described in the equation: $CL(L/kg/hr) = 0.587 + TEMG \times (-0.184)$. The CL of diclofenac in the hypothermic groups was estimated to be 68.7% of those in normothermia. The final pharmacokinetic model for dextromethorphan included a temperature (TEMG) covariate in central volume of distribution (V_1), which can be described as $V_1(L/kg) = 0.204 + TEMG \times (-0.0685)$. The central volume of distribution of dextromethorphan in hypothermia group was estimated to be 66.4% of that seen in normothermia. The final pharmacokinetic model for chlorzoxazone included a CAG covariate in CL, and TEMG covariate in peripheral volume of distribution (V_2). The CA groups showed decreased clearance of chlorzoxazone, which can be described as $CL(L/hr/kg) = 0.433 + CAG \times (-0.204)$. Systemic clearance of chlorzoxazone in the CA groups was estimated to be 52.9% of those in shams. In addition, hypothermia was associated with a decreased V_2 for chlorzoxazone, described as: $V_2(L/kg) = 0.970 + TEMG \times (-0.478)$. The peripheral compartment volume of chlorzoxazone in the hypothermia was estimated to be 50.7% of that seen in

normothermia. In addition, three physiological parameters (glucose, BUN and K) were tested individually as continuously variables, and no significant association was found with any of the PK parameters.

Visual predictive check

Predictive check from 1000 simulations using the final model estimates was performed for each model. The predict check curves demonstrated that the simulated distribution of median and 95% CI values were in agreement with the observed values (Figure 5). The simulation, as well as the diagnostic plots, revealed that the final models provided a reliable description of the data with good precision of the structure model estimates and covariate effect.

Possible CA-hypothermia interaction

The individual clearances of midazolam, diclofenac and chlorzoxazone from their final models were generated by NONMEM. Temperature and individual clearance correlations have been plotted in Figure 6. Within the same temperature groups, sham and CA have been separated. Two trend lines across sham normothermia-sham hypothermia and across CA normothermia-CA hypothermia were added separately (Figure 6). Possible temperature-CA interaction was found for clearances of chlorzoxazone when the two trend lines were not parallel with each other (not in the same trend). The interaction indicated that when both CA and hypothermia exist, the metabolism would be different than if either occurred alone.

DISCUSSION

Our study evaluated the effects of systemic hypothermia mediated alterations on the activity of multiple CYP isoforms *in vivo* after cardiac arrest. Our results have revealed that the magnitude of the CA and temperature effects are CYP isoform-specific with the greatest alterations in the metabolism observed for CYP3A and CYP2E1 isoforms. Our results suggested that the combination of hypothermia with CA was more likely to be associated with decreased enzyme activities. Although other reports have focused largely on the effects of hypothermia on drug metabolism, we believe that CA and temperature-CA interaction findings may have clinical implications for patients with CA in whom therapeutic hypothermia is used.

CA is more likely correlated with the decreased activity of CYP3A observed in the hypothermic CA group. CA and associated sequelae can cause severe metabolic disturbances. Surprisingly, these effects on hepatic drug metabolism are currently also poorly understood. Previous studies have shown that the metabolism and elimination of drugs are often altered in critically ill patients. Shelly *et al* demonstrated the failure of critically ill patients to metabolize midazolam. The clearance of midazolam was reduced and plasma concentration of its metabolite 1'-hydroxymidazolam was decreased in septic shock patients (Shelly et al., 1987). Spina *et al* has shown that therapeutic monitoring of midazolam in the ICU is warranted (Spina and Ensom, 2007). Kirwan *et al* showed that midazolam can be used to monitor changes in hepatic drug metabolism in critically ill patients. The concentration of midazolam at 4 hours after administration was significantly greater in critically ill patients as compared to normal renal function subjects (Kirwan et al., 2009). A previous study by Vree *et al* has reported the decreased elimination rate of midazolam in the intensive care patients, which may due to the decreased albumin and altered tissue binding (Vree et al., 1989). The CA effect as well as

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

Previous studies demonstrated reduced metabolism of CYP3A substrates during hypothermia (Caldwell et al., 2000; Fritz et al., 2005; Fukuoka et al., 2004; Hostler et al., 2010). Our previous study predicted around 11.1% midazolam clearance decrease per degree Celsius change below 36.5°C in healthy volunteers. Altered pharmacokinetic parameters including both clearance and volume of distribution have been reported during hypothermia. Hypothermia decreased the central compartment volume of midazolam in this study, and therefore also explained the observed higher plasma concentration during hypothermia. Our lab also previously tested midazolam metabolism during hypothermia after IV infusion, and there were around 20% decreases in clearance in hypothermic CA compared to those in normothermic CA, which was consistent with our current study. Our current understanding is that the combination of hypothermia and CA may both affect the activity of CYP3A and the metabolism and distribution of midazolam.

Similarly, the combination of hypothermia and CA affects the activity of CYP2E1. There was also a hypothermia-CA interaction effect on the clearance of chlorzoxazone. In addition, hypothermia reduced the peripheral volume of distribution. This result is consistent with our previous study in the lab, in which no significant difference observed between CA normothermia and CA hypothermia at 33°C for chlorzoxazone metabolism under five hours acute hypothermia effect (Tortorici et al., 2009). Twenty-four hours after CA and rewarming, the activity of CYP2E1 in CA normothermia group was found significantly lower compared with control and

CA hypothermia. Previous study in our lab has shown that hypothermia significantly reduced the CYP2E1 activity at 30°C post cardiac arrest (Tortorici et al., 2006). In addition, hypothermia reduced binding affinity (K_m) rather than capacity (V_{max}) for chlorzoxazone at 30°C *in vitro*. A previous study showed that the metabolism of chlorzoxazone and mephenytoin were correlated with the multiple organ dysfunction score and the multiple organ failure score, when they studied CYP2C9, CYP2C19 and CYP2E1 activity in critically ill trauma patients (Harbrecht et al., 2005). The CYP isoform activity was differentially altered has been suggested.

Furthermore, there was no significant difference found for diclofenac (CYP2C) clearance among four groups by non-compartmental model analysis. However, in population PK model, after we grouped hypothermia groups vs normothermia groups, and there was a weak association between hypothermia and reduced clearance of diclofenac. In this model, omega block structure (on CL, Q, and V2) was used which indicated an interaction between clearance and volume of distribution. This result may indicate a differential effect from mathematic calculation and the temperature effect on CYP2C activity needs further investigation. Similarly, there was no significant difference of clearance found for dextromethorphan (CYP2D) among the four groups. Hypothermia was correlated with the reduced central volume of distribution of dextromethorphan. During hypothermia, the volume of distribution of many drugs is affected, among which pentobarbital and rocuronium are CYP2D substrates (Schaible et al., 1982; Beaufort et al., 1995). Compared with other CYP isoforms, CYP2D as well as CYP2C can interact with specific functional groups in the substrate (e.g., a basic nitrogen for CYP2D6 and an acid for CYP2C9) and help orient them and possibility maintain the metabolic rate and the clearance of the substrates better than other CYP isoforms (Redlich et al., 2008). Potential mechanisms for altered volume of distribution during hypothermia include global blood

perfusion changes, blood pH alterations, altered lipid solubility, and potential alterations in drug tissue binding. Currently no studies have explored the exact mechanisms of these alterations, however, the reduction in volume of distribution has been consistent across studies and warrants future mechanistic evaluation.

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

One limitation of this study is the use of probe drugs as indices of given CYP phenotypic activities. Although the best possible probes were selected for this analysis, it is important to note the significant limitations of the use of these probes as phenotypic measures of CYP isoform specific activity. In particular, diclofenac has been shown in both rats and humans to

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

In conclusion, our current results have shown the effect of CA and hypothermia with interaction on isoform specific activity. Give the prominent role that the use of mild hypothermia in the management of patients with CA, further translational studies using clinically relevant drugs and pharmacokinetics-pharmacodynamics modeling are vital to further explain and confirm the isoform specific effect in different disease and temperature states. Ultimately, the effect of therapeutic hypothermia on drug metabolism in patients with CA must be further understood in order to optimize therapy in these highly vulnerable patients.

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Authorship Contributions

Participated in research design: Zhou, Empey, Kochanek, and Poloyac

Conducted experiments: Zhou

Contributed new reagents or analytic tools: Zhou, Poloyac

Performed data analysis: Zhou, Bies

Wrote or contributed to the writing of the manuscript: Zhou, Empey, Bies, Kochanek, and Poloyac

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Legends for Figures:

Figure 1: Temperature-time histories of the four treatment groups (Mean±SD). Dashed lines are hypothermia groups and solid lines are normothermia groups. Blank dots are CA groups and filled dots are sham groups. (● sham normothermia, ■ sham hypothermia, ○ CA normothermia, □ CA hypothermia).

Figure 2: Time course of plasma concentrations obtained for midazolam, diclofenac, dextromethorphan and chlorzoxazone from 0-8 hours after IV bolus 0.2 mg/kg midazolam, 0.5 mg/kg diclofenac, 0.5mg/kg dextromethorphan, and 0.5mg/kg chlorzoxazone. Dashed lines are hypothermia groups and solid lines are normothermia groups. (● sham normothermia, ■ sham hypothermia, ○ CA normothermia, □ CA hypothermia).

Figure 3: Systemic clearances of midazolam, diclofenac, dextromethorphan and chlorzoxazone in four groups by non-compartmental PK analysis. Significant difference between groups was tested using one-way ANOVA with Bonferroni post hoc testing. * $p < 0.05$

Figure 4: Goodness-of-fit diagnostic plots: Individual predicted concentration-Observed concentration and Weighted residuals-Time of final models of (A) midazolam, (B) diclofenac, (C) dextromethorphan and (D) chlorzoxazone from their final pharmacokinetic models. The solid line in plot is the line of unity.

Figure 5: Predictive check from 1000 simulations using the final estimated parameter of each model. (A) midazolam, (B) diclofenac, (C) dextromethorphan and (D) chlorzoxazone. Solid line is the median of all simulated concentrations. Dashed lines are 5th and 95th percentiles of all simulated values. All dots are observed values from the study. The simulated data and the observed data are in great agreement with each other.

Figure 6: Individual generated clearances vs temperature from final pharmacokinetic model for (A) midazolam, (B) diclofenac and (C) chlorzoxazone. Filled symbols (◆) with solid trend line are sham groups. Open symbols (◇) with dashed trend line are CA groups.

Table1: Physiological parameters at different time points in four groups

		Baseline before CA	1-hr after resuscitation	End of cooling induction, PK baseline	4-hour after cooling	8-hour after cooling
MAP	Sham normo	101.7±7.5	92.7±8.8	102.8±13.8	125.8±16.4	115±15.2
	Sham hypo	110.6±29.5	100.3±11.5	119.5±17.4	131.7±21.9	129.2±14.5
	CA normo	109±27.9	59.3±14.4†	86.3±21.3	110±5.8	136.5±11.9#
	CA hypo	88±11.5	69.2±16.8#†	138.6±12.9#†	130.5±20.5#	126.3±9.9#
PaCO2	Sham normo	37.9±5.9	36.4±4.4	35.8±4.2	35.2±3.4	34.8±4.4
	Sham hypo	37.1±4.5	37.2±2.4	40.1±5.1	36.8±7.1	30.8±3.7#
	CA normo	39.3±3.2	32.4±5.4#	36.7±4.1	39.6±8.0	35.4±5.3
	CA hypo	36.5±2.6	29.1±2.3 # †	49.7±5.9#†	42.9±5.5#†	36.9±8.3
Glucose	Sham normo	86.8±20.2	65.7±8.07#	69.7±10.9#	78.2±18.6	75.3±23.5
	Sham hypo	113.5±30.7	76.8±16.6#	87.8±19.1#	83.8±18.2#	89.2±17.4#
	CA normo	112±39.9	63.6±11.8#	56±5.5#†	85.2±28.2#	71±30.3#
	CA hypo	92.6±17.0	53±13.4#†	72.8±29.6#	80.7±16.7#	73.3±38.1#
Lactate	Sham normo	0.78±0.23	0.78±0.29	0.82±0.36	0.32±0.30#	0.5±0.25#
	Sham hypo	0.96±0.24	0.9±0.35	0.56±0.23#	0.62±0.23#†	0.4±0.34#
	CA normo	0.82±0.18	1.40±0.7†	0.7±0.4	0.8±0.23†	1.32±1.5#†
	CA hypo	0.96±0.31	0.88±0.48	0.25±0.13#†	0.47±0.25#†	0.38±0.11#†
BUN	Sham normo	24.7±7.3	22.5±4.5	23.5±6.0	38±12.7#	57.8±19.7#
	Sham hypo	23.2±5.6	24±3.8	23.2±4.6	37.2±10.3#	49.2±16.7#
	CA normo	26.5±8.3	30.1±6.2†	32.3±7.5#†	45±9.8#†	66.8±18.0#†
	CA hypo	26.8±7.9	32.2±9.0#†	31.7±5.7#†	46.2±10.2#†	60.6±16.8#
Hematocrit%	Sham normo	36.7±2.9	35.3±1.2	34.2±1.6	32.3±2.7#	35±1.0
	Sham hypo	35.8±1.6	35.4±2.4	35±1.0	31.6±1.0#	30.8±1.7#†
	CA normo	37±2.8	35.5±1.5	33.3±3.2#	30±1.9#	30.8±1.6#†
	CA hypo	37±4.3	36.8±2.1	37.5±1.6†	31±1.3#	32.8±3.7#†
pH	Sham normo	7.38±0.03	7.38±0.03	7.39±0.03	7.36±0.06	7.36±0.04
	Sham hypo	7.38±0.02	7.45±0.16	7.36±0.01	7.37±0.03	7.39±0.03
	CA normo	7.37±0.03	7.44±0.03	7.39±0.01	7.38±0.02	7.35±0.06
	CA hypo	7.39±0.03	7.43±0.03	7.29±0.04#†	7.32±0.05#†	7.33±0.03#
Mg2+	Sham normo	0.36±0.03	0.37±0.03	0.37±0.04	0.41±0.05	0.41±0.06
	Sham hypo	0.39±0.03	0.39±0.04	0.40±0.03	0.41±0.05	0.43±0.05
	CA normo	0.36±0.04	0.39±0.04	0.41±0.02	0.43±0.04#	0.46±0.06#†
	CA hypo	0.34±0.05	0.43±0.05#†	0.42±0.02#†	0.41±0.05#	0.46±0.09#†
K+	Sham normo	4.75±0.53	4.74±0.40	4.61±0.37	5.25±0.66#	5.62±0.98#
	Sham hypo	4.91±0.30	4.79±0.24	4.12±0.35	3.75±0.34#†	4.83±0.28
	CA normo	4.78±0.35	4.84±0.31	4.84±0.37	5.43±0.47	5.97±0.42#
	CA hypo	4.6±0.48	4.73±0.45	3.89±0.36#†	4.83±0.18	5.67±0.9#

#: $p < 0.05$ compared with the baseline time point within the same treatment group (compare within row), by one way repeated ANOVA. †: $p < 0.05$ compared with the sham normothermia within the same time point (compare within column) by one way repeated ANOVA.

Table 2: Pharmacokinetic variables of four treatments from non-compartmental analysis for midazolam, diclofenac, dextromethorphan and chlorzoxazone

PK parameters	Groups	AUC _{0-T} ($\mu\text{g}\times\text{min}/\text{mL}$)	AUC _{0-inf} ($\mu\text{g}\times\text{min}/\text{mL}$)	AUC _{extrap} %	V _z (L/kg)	Cl _s (mL/min/kg)
Midazolam	Sham normo	164±43	167±44	(0.39~1.3)	2.3±1.1	1268±349
	Sham hypo	230±139	234±144	(0.39~3.0)	1.5±0.75	1055±428
	CA normo	327±271	336±268	(0.28~14.8)	2.1±1.5	791±321
	CA hypo	321±128	329±131	(0.05~5.8)	1.4±1.3	682±190*
Diclofenac	Sham normo	838±364	873±361	(1.8~12.7)	1.5±0.91	647±226
	Sham hypo	1169±915	1195±941	(0.87~3.3)	0.99±0.5	580±273
	CA normo	818±368	852±359	(0.71~18.8)	1.9±1.1	660±235
	CA hypo	1252±551	1213±579	(2.9~7.3)	1.5±0.98	489±203
Dextromethorphan	Sham normo	782±431	869±269	(1.4~19.8)	1.8±0.87	633±226
	Sham hypo	675±109	702±106	(2.1~5.0)	2.7±2.4	677±292
	CA normo	748±299	786±304	(2.3~10.9)	2.1±1.1	706±228
	CA hypo	1192±811	1264±815	(1.5~12.9)	1.5±1.2	506±223
Chlorzoxazone	Sham normo	926±442	1112±742	(1.0~16.7)	2.4±2.3	562±216
	Sham hypo	1167±617	1449±262	(2.5~4.8)	0.83±0.2	355±66
	CA normo	1748±444	2055±521*	(6.3~19.7)	1.3±0.6	255±55*
	CA hypo	2254±835*	2409±863*	(0.2~12.7)	0.61±0.3	230±76*

One way repeated ANOVA with Bonferroni post-hoc has been used to detect the difference

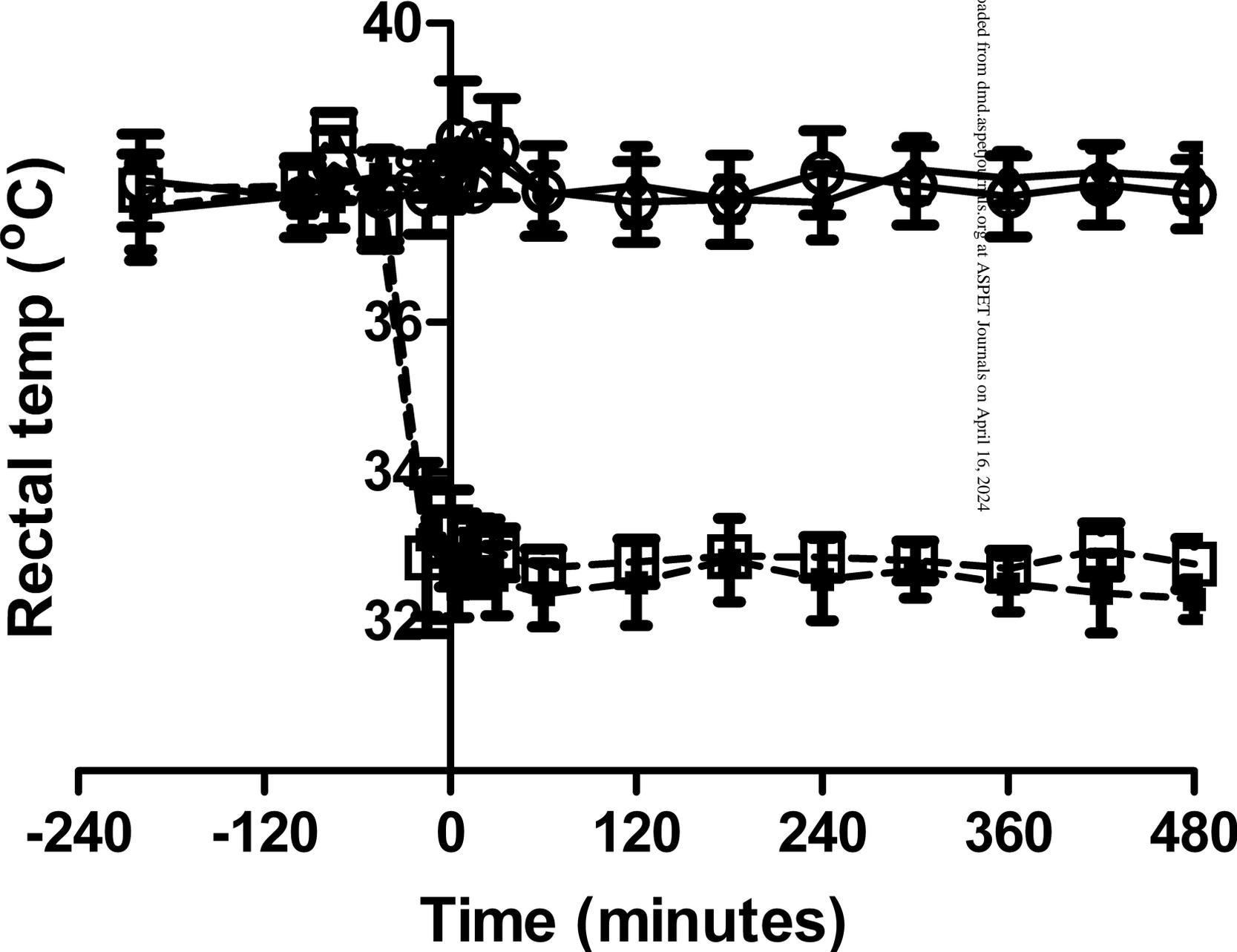
*among the four treatment groups. Data presented as mean (SD). *p < 0.05*

Table 3: Population pharmacokinetic parameter estimates (with standard error %) from nonlinear mixed effect model for midazolam, diclofenac, dextromethorphan and chlorzoxazone.

Parameters Estimate (se%)	Midazolam CL= $\theta_1 + \text{CAG} \times \theta_5$ V ₁ = $\theta_2 + \text{TEMG} \times \theta_6$	Diclofenac CL= $\theta_1 + \text{TEMG} \times \theta_5$	Dextromethorphan V ₁ = $\theta_2 + \text{TEMG} \times \theta_5$	Chlorzoxazone CL= $\theta_1 + \text{CAG} \times \theta_5$ V ₂ = $\theta_4 + \text{TEMG} \times \theta_6$
CL(θ_1)	0.990 (12.7)	0.587 (6.55)	0.504 (5.33)	0.433 (5.64)
V ₁ (θ_2)	0.258 (2.42)	0.138 (2.31)	0.204 (2.63)	0.142 (1.68)
Q(θ_3)	0.644 (10.6)	0.507 (8.22)	1.01 (12.5)	0.889 (10.6)
V ₂ (θ_4)	1.0 (11.9)	0.854 (15.1)	1.42 (15.5)	0.970 (15.1)
Covariate θ_5	-0.318 (11.5)	-0.184 (9.06)	-0.0685 (3.2)	-0.204 (6)
Covariate θ_6	-0.0786 (16.4)	—	—	-0.478 (15.8)
ω^2 (CL)	0.119 (6.21)	0.149 (5.48)	0.269 (11.3)	0.126 (3.54)
ω^2 (V ₁)	0.018 (14.6)	0.082 (12.4)	0.0385 (2.86)	—
ω^2 (Q)	0.460 (12.4)	0.312 (12.6)	0.234 (8.99)	0.218 (9.44)
ω^2 (V ₂)	0.214 (14.7)	0.406 (11.8)	0.170 (5.73)	0.170 (5.91)
σ^2	0.107 (12.5)	0.119 (1.87)	0.0838 (1.47)	0.0662 (0.877)
Final OFV	1268.8	1939.5	1880.9	2094.5
OFV change	10.4	6.0	4.7	23.6
p value	p<0.05	p<0.05	p<0.05	p<0.01

CL: clearance, L/hr/kg; V₁: central compartment of distribution, L/kg; Q: inter-compartment clearance, L/hr/kg, V₂: peripheral compartment of distribution, L/kg; CAG: CA categorical covariate, 0=sham, 1=CA; TEMG: temperature categorical covariate, 0=normothermia, 1=hypothermia; ω^2 : inter-individual variability; σ^2 : residual error; OFV: objective function value; Covariate (theta 5) is the effect of the first significant covariate on the specific PK parameters (CA for midazolam and chlorzoxazone, and temperature for dextromethorphan and diclofenac). Covariate (theta 6) is the effect of the second significant covariate found on specific PK parameters (temperature, for midazolam and chlorzoxazone).

Figure 1



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Figure 2

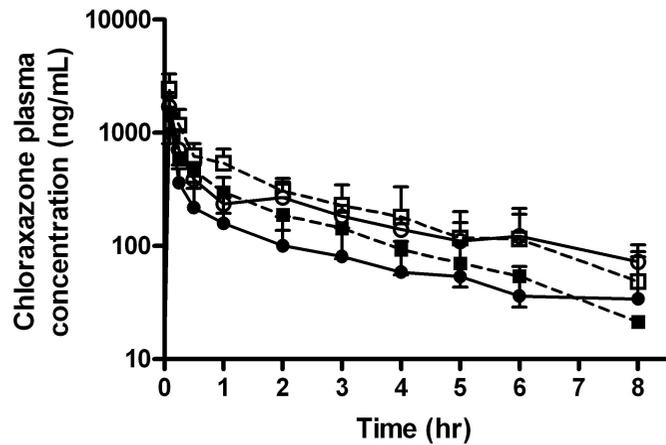
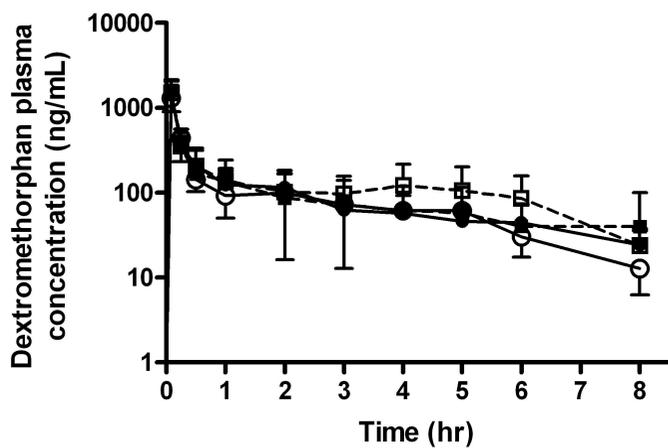
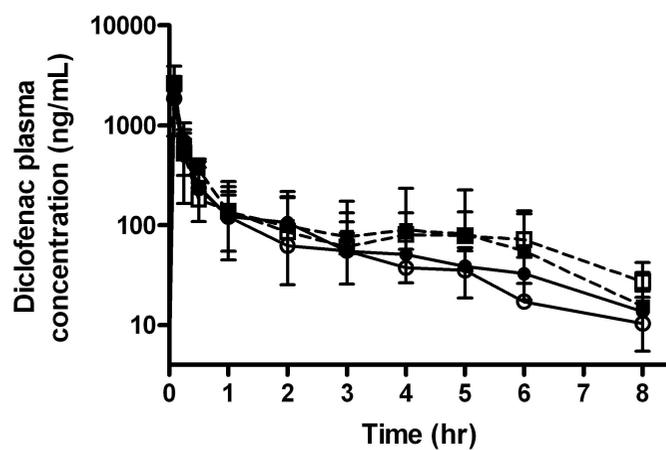
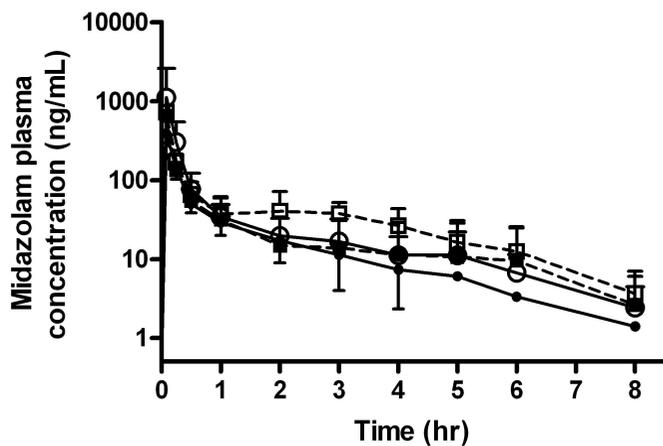


Figure 3

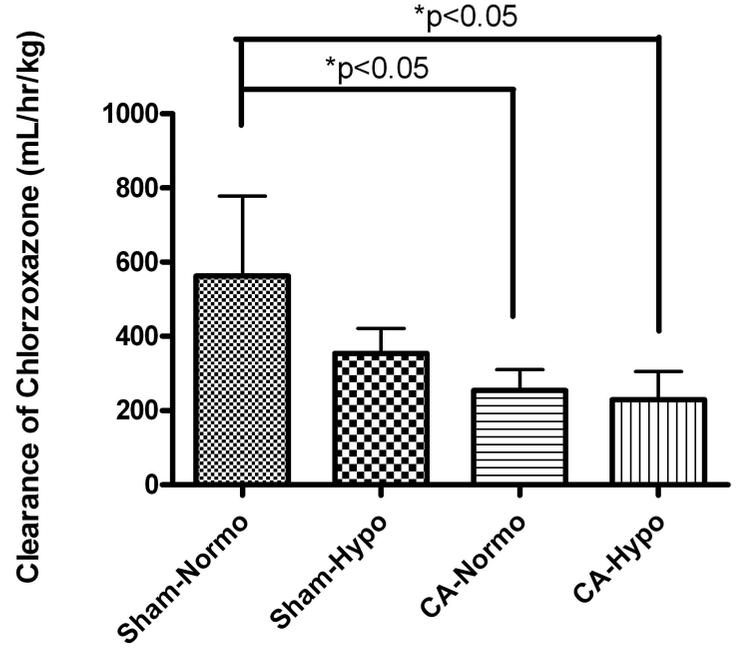
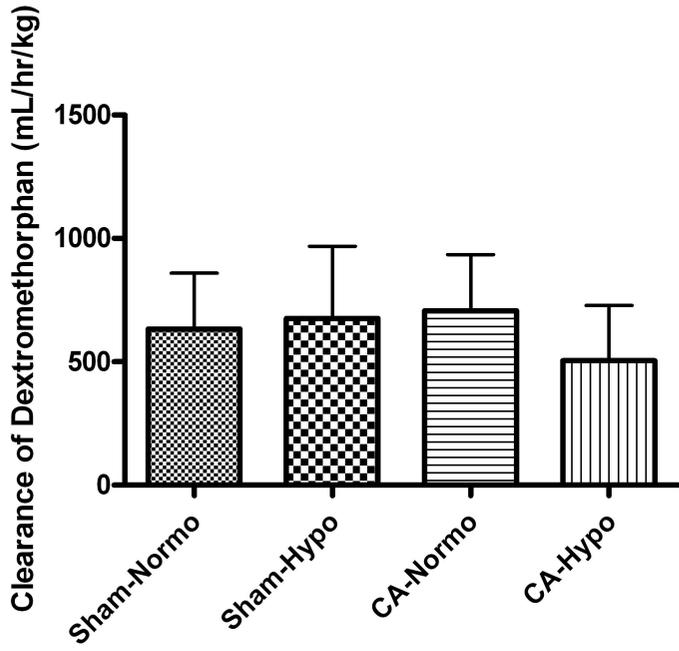
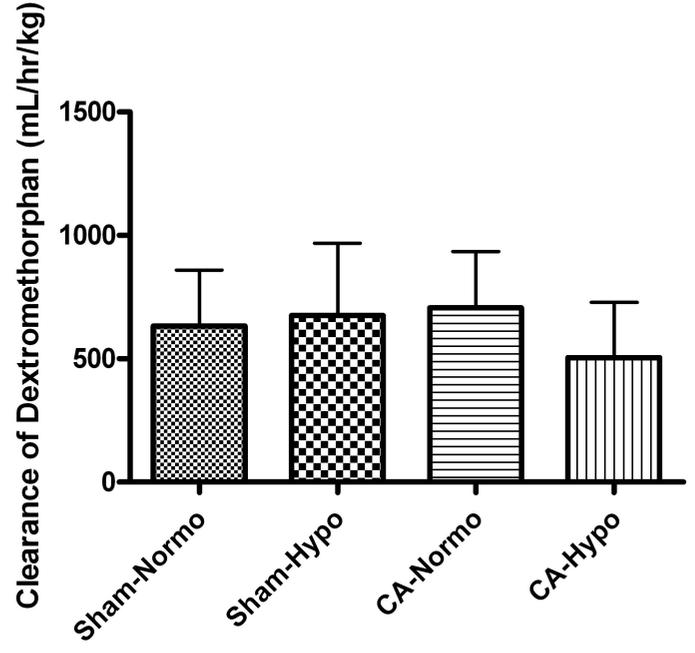
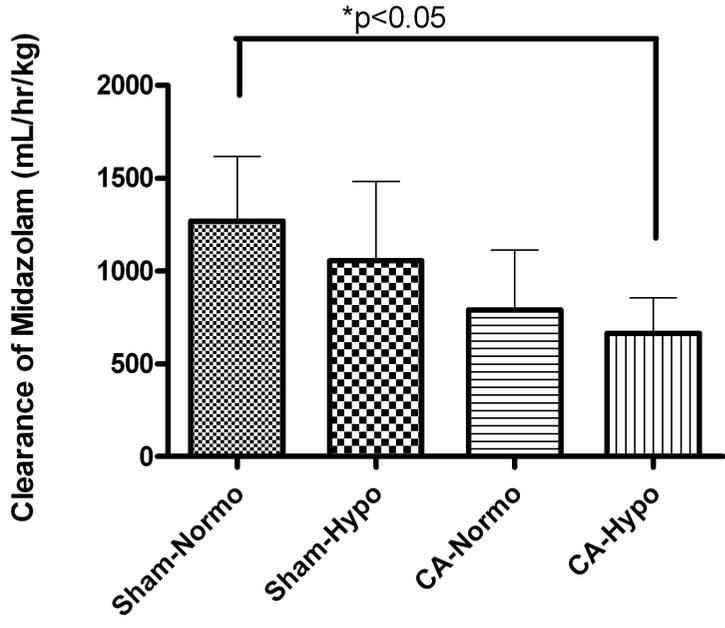
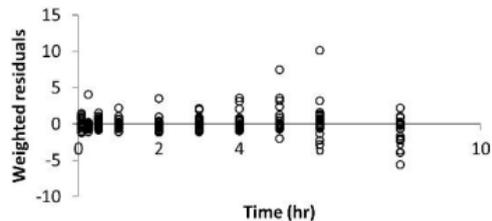
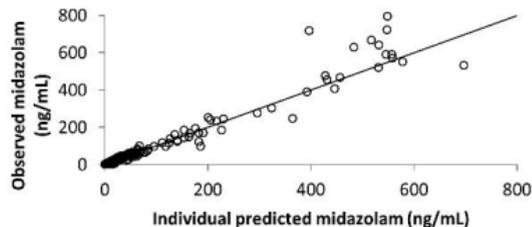
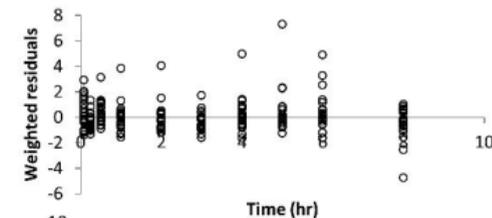
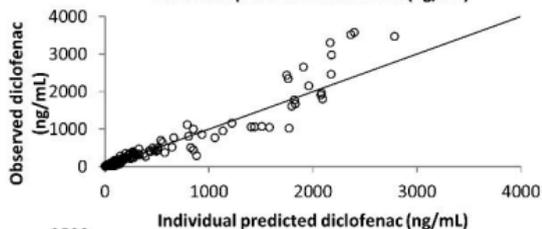


Figure 4

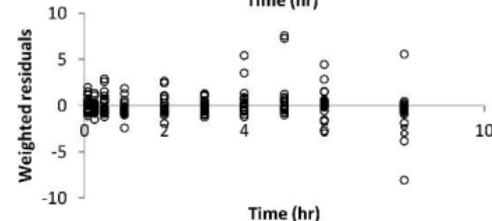
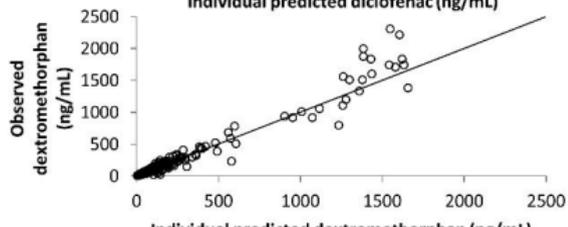
(A)



(B)



(C)



(D)

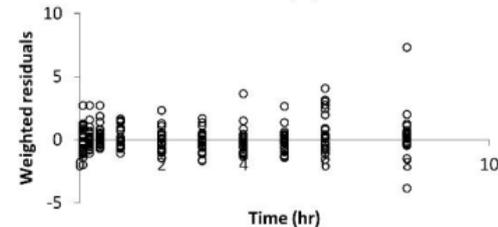
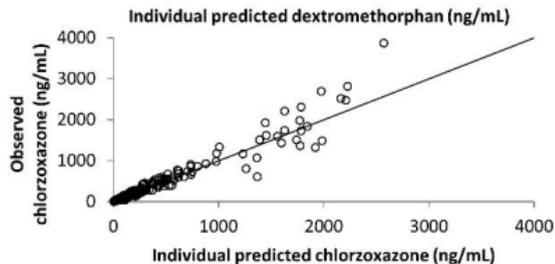


Figure 5

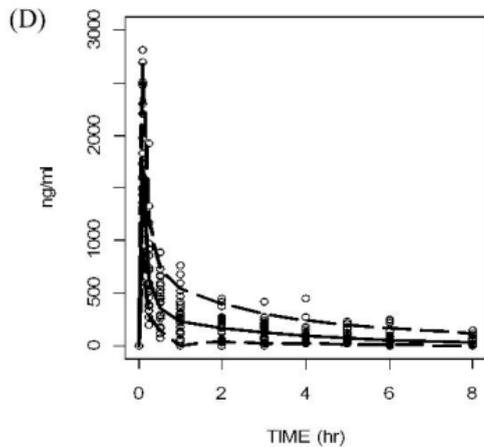
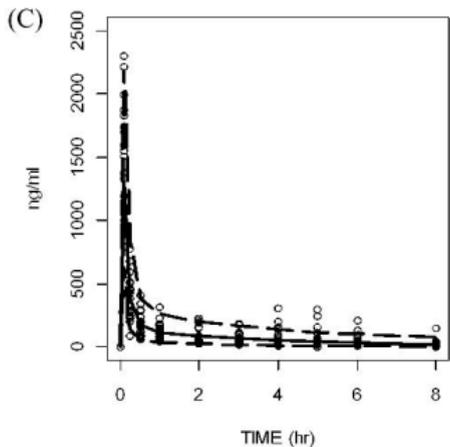
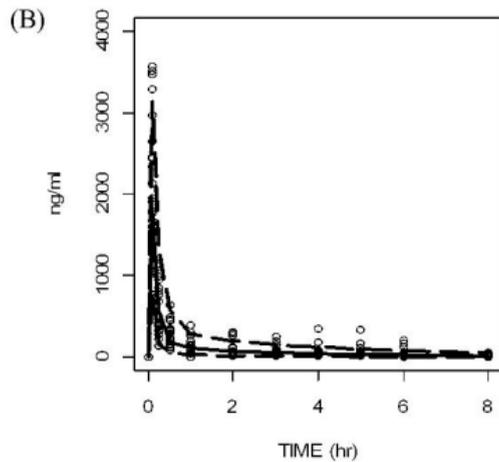
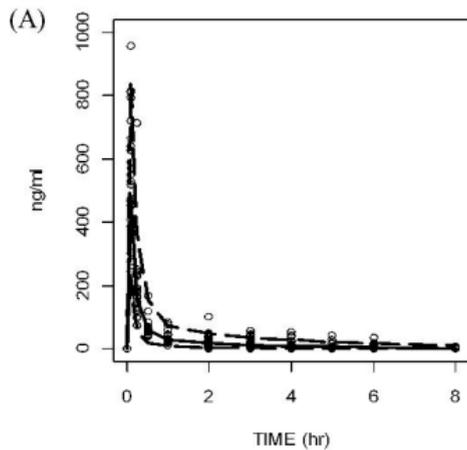


Figure 6

