Cross-Species Comparison of the Metabolism and Excretion of Zoniporide: Contribution of Aldehyde Oxidase to Interspecies Differences

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Running Title: Metabolism and Excretion of Zoniporide

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Text pages: 53
Tables: 6
Figures: 10
References: 24

Words in Abstract: 232
Words in Introduction: 574
Words in Discussion: 1476
Abbreviations

NHE-1, Sodium/Hydrogen exchanger; ADME, Absorption, distribution, Metabolism and Excretion; MTBE, Methyl t-butylether; RAM, Radioactivity monitoring detector; LC-ARC, Liquid Chromatography – Accurate Radioisotope Counting System;
Abstract

Excretion and metabolism of zoniporide was investigated in humans following intravenous infusion of [14C]zoniporide at 80 mg dose. Bile was the primary route of excretion since 57% of dose was recovered in the feces following IV infusion. Zoniporide was primary cleared via metabolism in humans. 2-Oxozoniporide (M1), was the major excretory and circulating metabolite in humans and was catalyzed by aldehyde oxidase (KM 3.4 µM; Vmax 74 pmol/min/mg protein). Metabolites M2 (17% of the dose) and M3 (6.4% of circulating radioactivity), in which the guanidine moiety was hydrolyzed to a carboxylic acid, were also detected in human feces and plasma respectively suggesting that hydrolysis was another route of metabolism of zoniporide in humans. The metabolism and excretion of [14C]zoniporide in rats and dogs was also evaluated. Like humans, bile was the primary route of excretion of the radiolabeled material in both the species and metabolism was the primary route of clearance. A comparison of plasma metabolites showed that for M3, rats had a higher concentration than human or dog. M1 was absent in dog and present in human and rat plasma at comparable levels, while comparison of excreta showed that the total body burden to M1 was greater in rat than in human. No further evaluation of M2 was considered since it was detected only in the human fecal extracts. Hence, no further toxicological evaluation of the three human metabolites was undertaken.
Introduction

Zoniporide (1-(quinolin-5-yl)-5-cyclopropyl-1H-pyrazole-4-carbonyl] guanidine) (Figure 1) was designed and synthesized as a highly selective inhibitor of sodium/hydrogen exchanger (NHE-1) (Tracey et al., 2003; Guzman-Perez, 2001; Marla et al. 2002; Knight et al. 2001) and was being developed to reduce the perioperative myocardial ischemic injury in high-risk surgery patients. It inhibited $^{22\text{Na}^+}$ uptake in fibroblasts expressing human NHE-1 in a concentration-dependent manner with an IC$_{50}$ of 14 nM and had >150-fold selectivity over other NHE isoforms (Marla et al. 2002). Zoniporide also showed reduction in infarct size and decreased the incidence and duration of potentially lethal arrhythmias following intravenous administration to animal models of ischemic-reperfusion injury (Knight et al. 2001; Tracey, et al. 2003).

Preclinical pharmacokinetic studies of zoniporide in mice, rats, rabbits, dogs, and monkeys demonstrated that its clearance (Cl$_p$) was moderate to high in all species. The volume of distribution (V$_d$) was moderate and the plasma t$_{1/2}$ was 1.5 h or shorter in all species evaluated. There was a linear increase in systemic exposure of zoniporide in healthy male human volunteers, following 1 h infusion of doses ranging from 0.003 to 1.0 mg/kg, as assessed by C$_{\text{max}}$ and AUC$_{0-\text{tlast}}$. The C$_{\text{max}}$ and AUC$_{0-\text{tlast}}$ ranged from 2 to 622 ng/mL and 2 to 930 ng-hr/mL, respectively, at these doses. The Cl$_p$ and V$_d$ were independent of dose, and the mean t$_{1/2}$ was approximately 3 h. The most common adverse events reported by subjects receiving zoniporide were somnolence and postural hypotension. Other adverse events included nausea, vomiting, and headache.

In drug development, it is important to study the metabolism and the routes of excretion of a drug candidate in humans. Further, assurance that all major circulating
metabolites observed in humans are present in at least one animal species used for safety assessment provides greater confidence that animal toxicology studies are relevant for human safety (Baillie et. al., Smith and Obach 2005; Smith and Obach 2006; Davis-Bruno and Atrakchi 2006; Atrachi 2009). The recent FDA guidance on metabolites in safety testing (FDA Guidance on Metabolites and Safety Testing, 2008) recommends that human circulating metabolites exceeding 10% of the parent should be present in at least equal quantities in at least one of the preclinical species used in toxicological assessment (Davis-Bruno and Atrakchi 2006, Atrakchi 2009). Direct testing in animals is warranted for human metabolites that are absent or present in disproportionately lower amounts in preclinical species used for toxicology studies.

Conventionally, an assessment of major metabolites in humans and preclinical species is accomplished by definitive ADME studies in which radiolabeled drugs are administered and biological fluids evaluated for a comprehensive and quantitative profile of metabolites. The current study was performed to compare the metabolism and excretion of zoniporide in humans and animal species used in toxicological testing using radiolabeled zoniporide. $[^{14}\text{C}]$Zoniporide (Figure 1) was administered to four young healthy male volunteers by intravenous infusion for 1 h and the excreta and plasma was collected to assess the mass balance, routes of excretion and circulating metabolites of zoniporide in humans. To assess the coverage of all human metabolites in toxicology species, $[^{14}\text{C}]$zoniporide was also administered to Sprague Dawley rats and Beagle dogs and the metabolites in the excreta and in circulation were also identified to determine the major metabolic routes of zoniporide. In addition, the characterization of the enzymes...
responsible for the formation of M1 in humans and the enzyme kinetics for the formation of M1 was also evaluated in vitro using human subcellular fractions in this study.
Methods and Materials

Reference Compounds, Radiolabel Zoniporide and Chemicals.

All synthetic standards for the metabolites were synthesized at Pfizer Global Research and Development (Groton, CT) using standard procedures. $^{14}$C]Zoniporide mesylate was synthesized by the radiochemistry group at Pfizer Global Research in Groton under GMP conditions. The label was located in the pyrazole ring of the molecule (Figure 1). The purity of the radiolabeled material was >99%. For animal studies, $^{14}$C]zoniporide (radiochemical purity 99.4%, specific activity was 26.6 mCi/mmol) was synthesized by the radiochemistry group at Pfizer Global Research in Groton. All other reagents and solvents used in the studies were of highest grade available and were obtained from commercial sources. Ecolite (+) scintillation cocktail was obtained from ICN (Irvine, CA). Carbosorb and Permafluor E+ scintillation cocktails were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA).

Study Design, Dosing and Sample Collection in Humans.

This was an open-label, single dose inpatient study conducted with four non-smoking healthy male human volunteers aged between 18 to 55 years and weighing between 74 to 79 kg. Before the study started, an institutional review committee approved the protocol and the informed consent document. All study participants gave written informed consent prior to initiation of the study.

All volunteers were administered a single 80 mg dose of $^{14}$C]zoniporide mesylate (100 μCi) by intravenous infusion over 1 h in water at a 10 mg/mL concentration. The specific activity of the dose was 1.25 μCi/mg. Urine was collected into containers surrounded by dry ice at predose (-8-0), 0-12, 12-24 and at 24 hr intervals during the
study through 144 hr post-dose (8 days). Feces was collected prior to dosing and then over 24 hr intervals up to 144 hr post-dose (8 days). The total weight of the urine and feces was recorded after each collection. Blood samples (sufficient to provide 6 ml of plasma) were collected for pharmacokinetic evaluation of total radioactivity and zoniporide at times 0 (just prior to dosing) and at 0.5, 1, 2, 4, 8, 12, 24, 36, 48 and 24 h after the start of the infusion. Subsequent samples were taken at 24 h intervals through 144 h. Additional blood sufficient to provide a minimum of 20 ml plasma was collected at 1, 4, 8, and 12 h post dose from the start of the infusion for characterization of metabolites. All samples were collected in heparinized tubes and centrifuged at approximately 4 °C. Samples were stored frozen until the day of analysis.

**Animal Studies.**

All studies were conducted in a research facility accredited by the American Association for the Accreditation of Laboratory Animal Care. All study animals were acclimated to standard housing and environmental conditions in metabolism cages and rooms where light cycles, temperature and humidity were documented daily for 2 days before the experiments.

**Rats:** Three male and three female Sprague-Dawley (SD) rats were housed individually in Nalgene metabolism cages and fasted overnight. A single dose [^14]C zoniporide was administered to all animals intravenously at a dose of 10 mg/kg containing 50 μCi radioactivity. The dose was prepared by dissolving [^14]C zoniporide in of 20% SBE-β-cyclodextrin at a concentration of 2.1 mg/mL. The specific activity of the dose was 28.8 μCi/mg. Urine was collected into containers surrounded by dry ice at predose (-24-0), 0-8, 8-24, and at 24 hr intervals during the study and feces over 24 hr intervals, up to 168 hr
post-dose. After the last urine and fecal collection, the cages were washed with 1:1 ethanol:water (v/v) and the wash was collected. The weight of each fecal sample, cage wash and the cage debris was determined. All animals were euthanized by CO₂ asphyxiation at the end of the study. For pharmacokinetic studies, six Sprague Dawley rats (n = 3/sex) were dosed intravenously with 10 mg/kg [¹⁴C]zoniporide. The dose was prepared by mixing unlabelled and radiolabelled zoniporide in 20% w/v SBE-β-cyclodextrin. The concentration and specific activity of the dose was 3.34 mg/mL and 7.46 μCi/mg respectively. Blood samples were collected in heparinized tubes at times 0 (just prior to dosing) 3, 15, 30, 60, 120, 240, 360 and 480 minutes. After sampling, whole blood was centrifuged at 14000 rpm for 3 min, plasma transferred to eppendorf snap capped tubes and stored at -20 °C until assayed. For identification of circulating metabolites, a separate group (2 animals, 1 per sex, per each time point) of animals were dosed intravenously at a dose of 10 mg/kg of [¹⁴C]zoniporide (containing 75 μCi of radioactivity). The specific activity of the dose was 30 μCi/mg. Blood was collected at 1, 2, 4, 8 and 24 h by sacrificing the rats at each sampling time. Samples were collected in heparinized tubes and centrifuged at approximately 4 °C. All samples were stored frozen at -20 °C until the day of analysis.

Dogs: Four non-cannulated (two per gender) Beagle dogs were housed individually in stainless steel metabolism cages and administered [¹⁴C]zoniporide by intravenous administration at a dose of 3 mg/kg (containing 150 μCi of radioactivity). The dose was prepared by dissolving the salt of unlabeled and labeled zoniporide (specific activity 26.6 mCi/mmol) into 20 ml of 20% SBE-β-cyclodextrin. The specific activity of the dose was 5 μCi/mg. Urine was collected in containers surrounded by dry ice at predose (-12-0), 0-
8, 8-24 and at 24 hr intervals during the study through 168 hr post-dose. Feces were collected prior to dosing and then over 24 hr intervals, up to 168 hr post-dose. The total weight of urine and feces was recorded after each collection. Blood samples were collected for pharmacokinetic evaluation of zoniporide and total radioactivity at times 0 (just prior to dosing) 0.5, 1, 2, 4, 8, 12, 24, and 24 hr intervals through 168 hr post-dose. For characterization of circulating metabolites, additional blood was collected at 0.5, 1, 2, 4, 8 and 12 hrs following an IV dose. Samples were collected in heparinized tubes and centrifuged at approximately 4 °C. All samples were stored frozen at -20 °C until the day of analysis.

Quantitation of Radioactivity.

Radioactivity in the plasma, urine and feces was determined by liquid scintillation counting. Aliquots of plasma (50 - 100 μL) and urine (100 - 500 μL) was counted in triplicate, by mixing with Ecolite (+) scintillation cocktail (6 mL) and counted in a Model L 1409 DSA liquid scintillation counter (Perkin-Elmer Wallac, Inc.). For determination of radioactivity in feces, the weight of each fecal sample was determined and the samples were homogenized in 2 parts of deionized water using a Stomacher blender 400. Following homogenization, triplicate aliquots (250-500 μL) of each sample were transferred into tared cones and pads, weighed and combusted in an automatic sample PerkinElmer 308 oxidizer (PerkinElmer, Boston, MA). The resulting 14CO2 was trapped in Carbo-Sorb (Packard Instruments, MA) and mixed with Perma-Fluor E+ (Packard Instruments, MA) scintillation fluid and the radioactivity was quantified by liquid scintillation counting. Radioactivity less than twice the background value was considered
to be below the limit of determination. Samples collected prior to dosing were used as controls and counted to obtain a background count rate.

The radioactivity in the plasma was expressed as ng-equiv. of zoniporide/mL. The compound equivalents were determined by dividing the μCi/mg sample by the specific activity of the compound (1.25 μCi/mg). Samples containing radioactivity (dpm) less than or equal to twice the background were considered to be zero in the calculation of the means. Radioactivity in the urine and feces was expressed as a percentage of the administered dose per time interval.

Quantitation of Zoniporide in Human, Rat and Dog Plasma.

Plasma samples were analyzed for zoniporide using a validated LC/MS/MS method. A 200 μL aliquot of each human plasma sample or a 50 μL sample from rat and dog plasma was treated with 100 μL solution of an internal standard (100 μg/mL of [15N3]zoniporide). The samples were basified with sodium carbonate solution and the analytes were extracted with MTBE. The organic layer was separated and evaporated to dryness and the residue was reconstituted with 200 μL of a mixture of acetonitrile and 2 mM ammonium acetate (15:85), the extracts were analyzed using a PE-Sciex API3000 mass spectrometer with a Turbo Ionspray source. The analytes were separated chromatographically using a Betabasic C18 column (100 x 2 mm, 5 μm) and detected in a positive ion mode using the protonated molecular ion as the precursor ion monitored at m/z 321.0 > 262.0 for zoniporide and at m/z 324.0 > 262.0 for the internal standard. Data collection and integration was performed using Sample Control and MacQuan™ Version 1.5 software (PE-Sciex, Concord, Ontario). Quantitation was based upon a linear least square regression analysis of calibration curves weighted 1/x² using the area ratio vs.
concentration. The dynamic range of the assay was 1.00 to 500 ng/mL for both zoniporide and M1.

**Determination of the Pharmacokinetic Parameters.**

Pharmacokinetic parameters were determined by non-compartmental methods from individual concentration time profiles after IV infusion using WinNonLin version 3.2 (Pharsight, Mountain View, CA). The maximum plasma concentration (C$_{\text{max}}$) and the time at which this concentration was achieved (T$_{\text{max}}$, at the end of the infusion) were directly taken from the concentration data. The area under plasma concentration versus time curve (AUC$_{0\text{-}\text{t}_{\text{last}}}$) was calculated from 0 to the last quantifiable time point (t$_{\text{last}}$), using log-linear trapezoidal approximation. The plasma terminal elimination rate constant (k$_{\text{el}}$) was estimated by linear regression analysis of the terminal slope of log plasma concentration – time curve. The terminal elimination half-life (t$_{1/2}$) was estimated as ln2/k$_{\text{el}}$. The total clearance (CL) was determined by the ratio; CL = Dose/AUC$_{0\text{-}\infty}$. The area under the plasma concentration – time curve from zero to $\infty$ (AUC$_{0\text{-}\infty}$) was estimated as the sum of AUC$_{0\text{-}\text{t}_{\text{last}}}$ and AUC$_{\text{t}_{\text{last}}\text{-}\infty}$. The AUC$_{\text{t}_{\text{last}}\text{-}\infty}$ was estimated from C$_{\text{t}_{\text{last}}}$/k$_{\text{el}}$, where C$_{\text{t}_{\text{last}}}$ represented the estimated plasma concentration at T$_{\text{last}}$, based upon the aforementioned regression analysis. The volume of distribution (V$_{\text{ss}}$) was determined by CL $\cdot$ MRT, with MRT being the mean residence time after iv infusion.

**Metabolic Profiling.**

*Urine:* Urine was pooled from 0-24 h so that $>90\%$ of the drug-related material excreted in urinewas accounted for. The pooled urine samples were lyophilized overnight and the residue was dissolved in 1 mL of water. The pooling was proportional
to the volume of urine collected at each time point. The aqueous layer was centrifuged and an aliquot (100 µL) was injected on to a column.

**Plasma:** Plasma samples obtained were pooled to account for >90% of the radioactivity using the method reported by Hamilton and co-workers for profiling of circulating metabolites (Hamilton et al. 1981). The pooled samples were treated with 5 parts of acetonitrile to 1 part of plasma. The mixture was then centrifuged and the supernatant was transferred to another tube. The pellets were washed once more to ensure that >90% of the radioactivity was recovered. The supernatants were mixed and evaporated to dryness in a turbovap at 35 °C under nitrogen. The residues were reconstituted in 100 µL of 5 mM ammonium formate (pH 3) and an aliquot (100 µL) was injected onto the column. Aliquots (30 µL) of the reconstituted samples were also counted on the liquid scintillation counter to determine the radioactivity extraction recovery.

**Feces:** Fecal homogenates were pooled on weight basis to account for 90% or greater of the drug-related material excreted in feces. Each pooled fecal sample was diluted with 30 mL acetonitrile and vortexed. The sample was then centrifuged and the supernatant was separated. The process was repeated several times until >90% of the radioactivity was extracted. All supernatants were mixed and evaporated to about 1 mL in a turbovap at 35 °C under nitrogen. The concentrated residue was extracted with 10 mL of hexane to remove all lipophilic material and the aqueous layer was evaporated to dryness in a turbovap at 35 °C under nitrogen. The residue obtained was reconstituted in ~300 µL of 3 mM ammonium formate solution and a small sample was analyzed by
liquid scintillation counting for radioactivity extraction recovery. A 100 µL aliquot of the reconstituted sample was injected onto the column.

**Separation, Quantification and Identification of Metabolites.**

Metabolic profiling was performed using the HPLC system that consisted of an HP-1100 membrane degasser, HP-1100 autoinjector and a HP-1100 binary gradient pump (Agilent Technologies, Palo Alto, CA). Chromatography was performed on a Zorbax C8 column (5 micron, 4.5 x 150 mm) by injecting 100 µL of the reconstituted sample. The mobile phase was initially composed of acetonitrile (solvent B) and 10 mM ammonium formate (pH 3.0) (solvent A). The flow rate was 1.0 mL/min and separation was achieved at ambient temperature. A 50 min gradient was used which was as follows: 0 to 10 min, 5% B; 10 to 20 min, 10% B; 20 to 35 min, 20% B; 35 to 40 min, 20% B; 40 to 44 min, 80% B and 44 to 45 min 5% B. The column was re-equilibrated at 5% B for the next 5 min prior to next injection. The post-column eluate was split such that 95% of the flow was monitored continuously with a RAM detector fitted with a liquid scintillation cell (IN/US, Riviera Beach, FL). The remaining 5% of the flow was diverted to the PE-Sciex API 3000 triple quadrupole mass spectrometer. The RAM was operated in the homogeneous liquid scintillation counting mode with the addition of 3 mL/min of Tru-Count scintillation cocktail (IN/US, Riviera Beach, FL.) to the effluent. The RAM response was recorded as a real time analog signal by the MS data collection system.

The metabolites in the urine and feces were quantified by measuring radioactivity in the individually separated peaks in the radiochromatogram using Winflow software (IN/US, Riviera Beach, FL.). The RAM provided an integrated printout in counts per minute and percentage of the radiolabeled material. The circulating metabolites were
detected by LC-ARC, (AIM Research Company, Wilmington, DE). The LC-ARC was operated in the homogeneous liquid scintillation counting mode with the addition of 2.5 mL/min of Tru-Count scintillation cocktail (IN/US) to the effluent. The circulating metabolites were quantified using the LC-ARC software (AIM Research Company, Wilmington, DE) by measuring the radioactivity in the chromatographically separated peaks.

The metabolites were identified using a PE SCIEX API 3000 mass spectrometer (PerkinElmerSciex Instruments, Boston, MA) equipped with an electrospray ion source operated in the positive ion mode. The instrument settings and potentials (e.g. collision energy) were adjusted to provide optimal data for zoniporide. The Sciex API 3000 was operated at an ionspray voltage (IS) of 4000 V and orifice voltage (OR) of 36 V. The collision induced dissociation (CID) studies (precursor ion scan or product ion scan) were performed using nitrogen gas at collision energy of 30 V and the collision gas thickness of $4 \times 10^{14}$ molecules/cm$^2$. The MS data was analyzed by MultiView 1.4 software (PerkinElmer-Sciex Instruments, Boston, MA). The metabolites were identified using Q1 (full scan), neutral loss, and precursor ion scanning techniques. The structures of metabolites were identified using product ion scan of the molecular ions that were identified in the above scanning modes and multiple reaction monitoring scanning.

**In Vitro Metabolism of Zoniporide.**

Zoniporide (10 μM), was incubated with human liver S-9 fractions (protein concentration 2.5 mg/mL), MgCl$_2$ (3.3 mM), in the presence of NADPH (3.0 mM) in a total volume of 1.0 mL potassium phosphate (0.1 M, pH 7.4). Incubations were started by addition of NADPH and shaken in a water bath set at 37 °C. Control experiments
were carried out in a similar manner except that the NADPH solution was substituted with phosphate buffer. After 1 h, the incubations were quenched with acetonitrile (5 mL), centrifuged and the supernatant was evaporated to dryness in a Turbo-Vap under nitrogen. The residue was reconstituted in 200 μL of acetonitrile and water mixture (1:3) and 25 μL aliquot was injected onto the column for the identification of the oxidation products. Enzyme phenotyping studies with zoniporide were carried out in a similar manner except that the incubation mixture first treated with raloxifene (10 μM), an inhibitor of aldehyde oxidase, prior to addition of the substrate.

The metabolites in the in vitro samples were identified by Finnigan LCQ Deca XP ion trap mass spectrometer (Thermo Fisher, Waltham, MA) equipped with an electrospray ion source. The mass spectrometer was operated in a positive ion mode and the operating parameters for the ion trap were as follows: capillary temperature 270 °C; spray voltage 4.0 kV; capillary voltage 4.0 V; sheath gas flow rate 90 and auxillary gas flow rate 30. The mass spectrometer was operated in a positive ion mode with data-dependent scanning. The ions were monitored over a full mass range of m/z 125-1000. For data dependent scanning, the normalized collision energy was 40%. The MS data was analyzed using the Xcalibur software version 1.4 (Thermoelectron, Waltham, MA).

**Enzyme kinetics.**

Zoniporide (1-500 μM) was incubated with pooled human liver cytosol (0.2 mg/mL; Sigma) and EDTA (0.1 mM) in 0.2 mL 25 mM phosphate buffer at 37 °C for 60 min. (Preliminary experiments demonstrated that the protein concentration and incubation time used provided linear reaction velocity.) The incubations were commenced with addition of cytosol and terminated with the addition of 0.05 mL 1M
formic acid. The terminated incubation mixtures were filtered through Millipore Multiscreen-HA filter plate (0.45 \( \mu \text{m} \)) and filtrates analyzed by HPLC-MS. Filtered incubation mixtures were injected (0.04 ml) onto a Phenomenex Luna C18 column (2.5 x 50 mm; 5\( \mu \); Phenomenex, Torrance, CA) preequilibrated in 0.1% HCOOH containing 5% CH\(_3\)CN at a flow rate of 0.4 ml/min. The HPLC system consisted of two Shimadzu LC-10AD pumps (Shimadzu; Columbia, MD), a CTC PAL autoinjector (CTC Analytics, Carrboro, NC), and a Micromass Ultima tandem quadrupole mass spectrometer (Micromass, Beverly, MA) operated in the multiple reaction monitoring mode. The mobile phase was maintained at initial conditions for 0.5 min followed by a linear gradient to 70% CH\(_3\)CN at 3 min. The effluent was introduced into an ionspray source in positive mode and tune file parameters were as follows: capillary: 3.5; cone: 25; source temperature: 135°C; desolvation temperature: 350°C; cone gas: \( \approx 190 \); desolvation gas: \( \approx 750 \); entrance: -5; collision: 20; exit: 1, with other potentials optimized to maximize the signal. Metabolite M1 (R\(_t\) = 1.98 min) was monitored with the transition \( m/z \) 337 to 278

Quantitation was accomplished by extrapolation from a standard curve ranging from 0.1 to 10 \( \mu \text{M} \). Assay standards were within +/- 9% of nominal values.

Since the initial velocity versus the substrate concentration plot showed a decrease in the rates at higher substrate concentrations, the enzyme kinetic data were fit to the uncompetitive substrate inhibition model using the enzyme kinetics module of SigmaPlot (v9.01):

\[
v = \frac{V_{\text{max}} \cdot [S]}{K_M + [S] + \left( \frac{[S]}{K_{\text{si}}} \right)}
\]

Equation 1
in which $V_{\text{max}}$ and $K_M$ are the terms for the standard Michaelis-Menten parameters maximum reaction velocity and substrate concentration at half-maximum velocity, and $K_{si}$ is the constant describing the substrate inhibition interaction (Houston and Kenworthy 2000). The intrinsic clearance value was then determined from the $V_{\text{max}}$ and $K_M$ using equation 2

$$C_{\text{int}} = \frac{V_{\text{max}}}{K_M} \quad \text{Equation 2}$$

IC$_{50}$ value was determined from:

$$\% \text{ of control activity} = 100 \cdot \left( A - \frac{B \cdot [I]}{[I] + IC_{50}} \right) \quad \text{Equation 3}$$

in which the terms $A$ and $B$ represent maximum and minimum control activities at $[I]=0$ and $[I]=\infty$, respectively.
Results

Excretion Studies:

Healthy Human Volunteers: Urine and feces was collected over 144 h (6 days) from four healthy male volunteers following intravenous infusion of 80 mg of $[^{14}\text{C}]$zoniporide for 1h. A mean 91% of the dose was recovered in the urine and feces over 144 h (Table 1). Majority of dose (>95%) was excreted within the first 96 h following administration of the radioactive dose in all subjects (Figure 2A). Bile was the major route of excretion since 57% of the dose was excreted in the feces following an intravenous infusion of $[^{14}\text{C}]$zoniporide (Table 1). About 34% of the total dose was excreted in the urine (Table 1).

Rats: A mean 89% of the dose was recovered after a single bolus 10 mg/kg dose of $[^{14}\text{C}]$zoniporide to Sprague Dawley rats (Table 1). Most of the radioactivity was recovered in the initial 48 h in all rats (Figure 2B). Like humans, majority of the dose (61%) was excreted in the feces of rats and only 28% of the dose was excreted in the urine after 168 h. This suggested that bile was the primary route of excretion of zoniporide material in rats as well.

Dogs: About 89% of the dose was recovered in the urine and feces of dogs after 168 h after an intravenous administration of $[^{14}\text{C}]$zoniporide at a dose of 3 mg/kg. Bile was the primary route of excretion in dogs as well since 62% of the administered dose was excreted in the feces of this species while 27% of the dose was recovered in the urine of dogs (Table 1). As in rats most of the dose was recovered in the first 48 h after dosing of the radiolabeled compound (Figure 2C).

Pharmacokinetics of total radioactivity and zoniporide:
Pharmacokinetics in Healthy Human Volunteers: The mean plasma concentration versus time profiles of total radioactivity and zoniporide following intravenous infusion of $[^{14}\text{C}]$zoniporide in healthy male volunteers are presented in Figure 3A. The $C_{\text{max}}$ of zoniporide (590 ng/mL) was about 66% of the $C_{\text{max}}$ of total radioactivity (899 ng-equiv/mL) and occurred at the end of infusion (1 h) of $[^{14}\text{C}]$zoniporide (Table 2). The $AUC_{(0-\text{last})}$ of zoniporide (833 ng-h/mL) accounted for ~28% that of the $AUC_{(0-\text{last})}$ of total circulating radioactivity (2925 ng-equiv.-h/mL). The half-life of total radioactive material was 6.7 h. Unchanged zoniporide was rapidly cleared in humans after intravenous infusion of $[^{14}\text{C}]$zoniporide with a clearance of 21 mL/min/kg and a half-life of 2.0 h (Table 2).

Rat: Figure 3B depicts the mean plasma concentration versus time profile for total radioactivity, unchanged zoniporide following single bolus intravenous administration of $[^{14}\text{C}]$zoniporide to rat. Like humans, zoniporide was rapidly cleared from rats ($Cl = 113$ mL/min/kg) with a half-life of 0.4 h (Table 2). The $AUC_{0-\text{last}}$ of unchanged zoniporide (1390 ng-h/mL) accounted for 39% of $AUC_{0-\text{last}}$ of total radioactivity (3570 ng-equiv.-h/mL) in rats (Table 2). The mean terminal elimination half-life of total circulating radioactivity was 2.0 h and was 5 fold longer than zoniporide.

Dog: After a bolus intravenous administration of 3 mg/kg of $[^{14}\text{C}]$zoniporide, the mean $AUC_{0-\text{last}}$ of total radioactivity and unchanged zoniporide was 3160 ng-equiv-h/mL and 2009 ng-h/mL, respectively (Table 2). The plasma concentration versus time profile for the total radioactivity and unchanged zoniporide is shown in Figure 3C. Thus, in contrast to rats and humans, the exposure of unchanged zoniporide accounted for 64% of circulating total radioactivity in dogs. Further, in contrast to rats and humans, the
DMD #030783

clearance of zoniporide was moderate (21 mL/min/kg) while the Vdss was higher (3.7 L/kg) in dogs. The mean terminal elimination half-lives of total circulating radioactivity and unchanged zoniporide were similar (2.8 and 2.5 h, respectively).

Metabolite Profiles of Zoniporide in the Urine and Feces

Healthy Male Volunteers: Only two metabolites (M1 and M2) were detected in the urine and feces of humans (Figure 4). Metabolite M1 was the primary metabolite detected in the excreta (52% of the total dose) while zoniporide and metabolite M2 represented 18 and 17% of the total dose (Table 3).

Rat: Representative HPLC-radiochromatograms of the extracted urinary and fecal (pooled from 0 - 24 hr) samples from rat are shown in Figure 5. Like humans, M1 was the primary excretory metabolite in rat and represented 35 % of the total dose (Table 3). Unchanged zoniporide also constituted majority of the dose in the urine and feces and accounted for 40% of the dose. The other metabolites M3 to M7 were minor metabolites and represented 0.8 to 5.9 % of the dose (Table 3).

Dog: Unlike the rat and humans, unchanged zoniporide was the primary constituent in the excreta of dogs (Figure 6) and accounted for 37% of the total dose) (Table 3). Major metabolites were M8 and M10 which accounted for 13 and 18% of the total dose, respectively. Other minor metabolites M3, M5, M7 and M9 detected in the excreta of the dog accounted for 8.6, 5.4, 4.1 and 2.1% of the total dose, respectively (Table 3). Most of these metabolites were excreted in the feces suggesting biliary excretion of the radiolabeled material.

Circulating Metabolites of Zoniporide
Human Circulating Metabolites: Circulating metabolites in humans were profiled by pooling the plasma samples obtained over 0 to 12 h using the method described by Hamilton and co-workers (Hamilton, et. al 1981) (Figure 7A). This method gave a good assessment of the overall exposure of each metabolite over 0-12 h. About 99% of the total circulating radioactivity could be identified. Three radioactive peaks were observed in plasma and corresponded to M1, zoniporide and metabolite M3 (Figure 7A). Metabolite M1 was the primary circulating metabolite and accounted for 60% of the circulating radioactivity whereas zoniporide and M3 accounted for 30 and 6.4% of the total circulating radioactivity (Table 4).

Rat: Circulating metabolites in the rat were identified after pooling the plasma samples obtained from 0 to 24 h according to the Hamilton pooling method. All circulating radioactivity in the rat plasma was accounted for. A representative HPLC-radiochromatogram of rat plasma is shown in Figure 7B. Of the four radioactive peaks identified in the plasma, zoniporide and M1 constituted 36 and 46% of the circulating radioactivity while the other metabolites, M3, and M6 constituted 9.0 and 8.5% of the circulating radioactivity, respectively (Table 4).

Dog: The HPLC radiochromatogram of circulating metabolites in plasma pooled from 1 - 12 h is shown in Figure 7C. At least 99% of the total circulating radioactivity in the pooled plasma was identified. Four radioactive peaks including zoniporide were observed in dog plasma. Zoniporide was the major component circulating and accounted for 62% of the circulating radioactivity (Table 4). The major circulating metabolite was M9 and represented 23% of the total circulating radioactivity, while other metabolites...
M3, M4, M8 and M10, accounted for 2.3 to 5.1% of the circulating radioactivity (Table 4).

**Assessment and Comparison of Exposures of Human Circulating Metabolites in Toxicology Species**

Both metabolites M1 and M3 detected in the plasma of humans were greater than >10% of the parent as measured by the ratio of the % radioactivity of the metabolite to the % of radioactivity of the parent drug as observed in human circulating metabolic profile. Therefore, in accordance with the guidance, it was important to determine if the exposure of these two metabolites was greater or similar to the exposure in the toxicology species. In order to assess the coverage of M1 and M3, the exposure of these two metabolites in humans and preclinical species were estimated by equation 4.

\[
\text{AUC}_{(0-t_{\text{last}})\text{metabolite}} = \% \text{ of Metabolite} \times \text{AUC}_{(0-t_{\text{last}})\text{radioactivity}}
\]

Equation 4

The estimated exposures of all metabolites are depicted in Table 4. As shown in Table 4, the exposures (AUC\((0-t_{\text{last}})\)) of M1 and M3 were 1755 and 187 ng-equiv.-h/mL respectively, in humans and the estimated exposures of M1 and M3 in the rat were 1642 and 321 ng-equiv.-h/mL respectively. Metabolite M1 was not present in the dog however, the estimated AUC\((0-t_{\text{last}})\) of M3 in this species was 73 ng-equiv.-h/mL. The estimated exposures of other metabolites observed in the rat and dog are also shown in Table 4. Determination of circulating levels of zoniporide using this method resulted in AUC\((0-t_{\text{last}})\) of 878, 1285 and 1959 ng-equiv.-h/mL in humans, rat and dog respectively and were within the range of experimental error when compared to the levels that determined by a validated bioanalytical method (Table 2).
To further confirm the coverage of M1 and M3 in the preclinical species, the exposures of these two metabolites were also estimated using equation 5 and the results are depicted in Table 5A.

\[
\text{Estimated Exposure}_{\text{metabolite}} = \left(\frac{\% \text{ Radioactivity}_{\text{metabolite}}}{\% \text{ Radioactivity}_{\text{parent}}}\right) \times \text{AUC}_{\text{zoniporide}}
\]

Equation 5

Since both human circulating metabolites M1 and M3, were present in disproportionately less amounts in the dog, an assessment of the coverage of exposure of these two metabolites was made only in the rat. Using equation 2, the exposure of M1 and M3 was 1666 and 178 ng.equiv.-h/mL respectively, in humans and 1776 and 348 ng.equiv.-h/mL, in the rat.

Assessment of coverage of M1 was also made by comparing the amount of metabolite excreted in the urine and feces of humans and rats, which provides a comparison of total body burden. The amount of M1 excreted in the urine and feces was determined using equation 6;

\[
\text{Amount of M1 (µg/kg)} = \text{Dose (mg/kg)} \times \% \text{ M1 in the excreta}
\]

Equation 6

As shown in Table 5B, the amount of M1 in the excreta of humans after a 1.14 mg/kg (assuming a 70 kg weight for humans) and rats after a 10 mg/kg dose was 594 and 3500 µg/kg, respectively. Thus, even though the circulating exposure to M1 in the rat is slightly lower than in human, the total body exposure in rat is actually greater.

**Identification of Metabolites:**

The structures of metabolites were elucidated by LC-MS/MS using a combination of full, precursor ion and neutral loss scanning techniques. All metabolites were further characterized using the product ion scans of the identified masses. Zoniporide gave a
signal at m/z 321 [M+H]^+ in a positive ion mode. The product ion mass spectrum of m/z 321 gave characteristic major fragment ions at m/z 262 (loss of guanidine) and 234 (further loss of carbon monoxide) and 220 (loss of the cyclopropyl ring) (Table 6). Wherever possible, the structures of the major metabolites were confirmed by comparing their retention time and mass spectra to the synthetic standards. The structures/proposed structures of metabolites of zoniporide are shown in Figure 8.

**Metabolite M1:** Metabolite M1 showed a molecular ion at m/z 337 which was 16 amu higher than zoniporide suggesting oxidation of the parent compound. The mass spectrum of m/z 337 gave a fragment ion at m/z 278, 250 and 236 (Table 6). All these ions were 16 amu greater than those observed in the mass spectrum of zoniporide. This suggested that the quinoline or the pyrazole moiety of the molecule was the site of metabolism. That the site of hydroxylation was the quinoline ring and M1 was 2-oxozoniporide was confirmed by comparing its retention time and mass spectrum with the authentic standard of 2-oxozoniporide.

**Metabolite M2:** Metabolite M2 gave a molecular ion at m/z 296. The mass spectrum of M2 at m/z 296 resulted in fragment ions at m/z 278 and 250 (Table 6). The ion at m/z 278 was a result of loss of water from M2 and the fragment ion at m/z 250 was a result of a loss of 46 amu from m/z 296. The loss of 46 amu which is characteristic of a carboxylic acid group from the molecule, suggested that M2 was a carboxylic acid derivative. The presence of the carboxylic acid was further confirmed by esterification with methanolic HCl to the corresponding methyl ester (data not shown). The mass spectral fragmentation also indicated that the molecular ion of M2 was 16 amu higher than that of M3 (see below). Overall, the above data indicated that M2 was a carboxylic
acid analog of hydroxylated metabolite of zoniporide. The position of hydroxyl group on the molecule and its structure was further confirmed by comparing its retention time and mass spectrum with the synthetic standard of M2.

**Metabolite M3:** Metabolite M3 gave a signal at \( m/z \) 280 (41 amu less than parent). A mass spectrum of M3 at \( m/z \) 280 showed major fragment ions at \( m/z \) 262 and 234 suggesting a loss of a water molecule followed by a loss of carbonyl functionality (Table 6). A difference of 46 amu between \( m/z \) 280 and \( m/z \) 234 suggested a loss of formic acid, which was characteristic of a carboxy group. Esterification of the metabolite using methanolic HCl resulted in the formation of methyl ester further confirming the presence of the carboxylic acid group in the molecule. Further these ions were similar to those observed in the mass spectrum of zoniporide suggesting that the guanidine moiety of zoniporide was modified (Table 6). Together the above data suggested that M3 was a carboxylic acid derivative of zoniporide. Comparison of the retention time and mass spectrum of M3 with that of the authentic standard of carboxylic acid further confirmed its structure.

**Metabolite M4:** Metabolite M4 showed a molecular ion at \( m/z \) 337 which was 16 amu greater than zoniporide. The mass spectrum of \( m/z \) 337 resulted in fragment ions at \( m/z \) 320, 278, 261 and 250 (Table 6). The fragment ions at \( m/z \) 278 and 250 were similar to those observed in the mass spectrum of M1 and resulted from the loss of guanidine (59 amu) followed by the loss of the carbonyl moiety (28 amu) suggesting that the addition had occurred on the pyrazolo-quinoline moiety. The fragment ions at \( m/z \) 320 and 261 resulted from the loss of 17 amu (a hydroxyl group) from \( m/z \) 337 and 278, respectively and characteristic of an N-oxide. Treatment of the samples with 33% titanium chloride
(TiCl₃) (Kulanthaivel et. al., 2004) resulted in the disappearance of the peak which suggested that the metabolite was an N-oxide of zoniporide. The exact location of the oxygen atom (the quinoline nitrogen or pyrazole nitrogen) could not be discerned from the data. However, since previous studies have shown that quinoline containing molecules can also undergo oxidation of the quinoline ring nitrogen in addition to the ring (Ehlhardt et. al. 1998), the structure of M4 was proposed as the quinoline N-oxide of zoniporide. No further studies were attempted to isolate and fully characterize the metabolite.

Metabolite M5: Metabolite M5 showed a protonated molecular ion at m/z 353, which was 32 amu greater than the molecular ion of zoniporide (m/z 321). Addition of 32 amu to the parent suggested further dihydroxylation of zoniporide. The mass spectrum of the metabolite showed major fragment ions at m/z 336, 294, 277, 266 and 249 (Table 6). The fragment ion at m/z 294 and 266 indicated a loss of 59 amu (loss of the guanidine moiety) followed by the loss of a carbonyl group suggesting that the pyrazoloquinoline moiety of zoniporide was dihydroxylated. Other fragment ions m/z 277 and 249, observed in the CID spectrum indicated a loss of 17 amu (hydroxyl group) from m/z 294 and 266 respectively. Since the loss of 17 amu is characteristic of an N-oxide as discussed above, M5 was proposed as an N-oxide of hydroxyzoniporide. No further characterization of this metabolite was done to ascertain the position of the hydroxyl group since the metabolite was present only in the preclinical species.

Metabolite M6: Metabolite M6 showed a protonated molecular ion at m/z 513, which was 176 amu higher than m/z 337, the hydroxylated zoniporide. This suggested glucuronidation of hydroxyzoniporide. The mass spectrum of m/z 513 gave a major
fragment ion at \( m/z \) 337 suggesting a loss of 176 amu from the molecular ion and a
fragment ion at \( m/z \) 278 suggesting further loss of guanidine. Based on this data, M6 was
identified as the glucuronide conjugate of hydroxyzonporide. The position of the
hydroxy group could not be assessed from this data. No attempt was made to further
characterize this metabolite since it was absent in the human matrices.

**Metabolite M7:** Metabolite M7 showed a protonated molecular ion at \( m/z \) 337
suggesting hydroxylation of zoniporide. The fragment ions in the mass spectrum of the
metabolite were similar to those observed in the mass spectrum of metabolite M1 (Table
6). The difference in the retention time of M1 suggested that M7 was a regio-isomer of
M1 however the exact position of the hydroxyl group could not be determined from the
mass spectrum. Since the oxidative metabolite was lacking in humans no further attempt
to determine the position of hydroxylation in the metabolite.

**Metabolite M8:** Metabolite M8 showed a molecular ion at \( m/z \) 355. The
molecular ion of M8 indicated an addition of 34 amu to \( m/z \) 321, a molecular ion of
zoniporide. The mass spectrum of \( m/z \) 355 gave fragment ion at \( m/z \) 296 (a characteristic
loss of the guanidine moiety) and at \( m/z \) 296, 278, 254 and 250 (Table 6). The loss of 59
amu that resulted in \( m/z \) 296 indicated that the guanidine moiety was intact. The
fragment ion at \( m/z \) 278 and 250 possibly resulted from loss of water from \( m/z \) 296
followed by a loss of the carbonyl group (28 amu). The mass spectrum also showed ions
at \( m/z \) 254 and 236 suggesting a loss of 42 amu (a cyclopropyl group) from \( m/z \) 296 and
278 respectively. The loss was similar to that observed in the CID spectrum of
zoniporide and suggested that pyrazoloquinoline group was the site of modification.
Together, these fragment ions indicated that the M8 was probably a dihydrodiol
metabolite of zoniporide (Figure 8). Previous studies with quinoline containing compounds have indicated that these motifs are capable of undergoing conversion to a dihydrodiol (Nicoll-Griffith et al., 1993). The definitive identification and characterization of the exact position of the dihydrodiol on the quinoline ring was not attempted.

Metabolite M9: Metabolite M9 showed a molecular ion at \(m/z\) 531, which was 176 amu higher than M8, indicating glucuronidation of M8. The mass spectrum showed fragment ions at \(m/z\) 472, 355, 296, 278, 250 (Table 6). The fragment ion at \(m/z\) 472 was a characteristic loss of the guanidine moiety suggesting that this group was not modified. The fragment ion at \(m/z\) 355 resulted from the loss of the glucuronide moiety. The remaining fragment ions were similar to those in the mass spectrum of M8. Previous studies have demonstrated that dihydrodiol metabolites of aromatic rings are capable of undergoing glucuronidation (Lantz et al., 2003). Thus, M9 was assumed to be a glucuronide conjugate of M8 (Figure 8). No further characterization of this metabolite was performed since it was not detected in the human metabolic profiles.

Metabolite M10: Metabolite M10 also showed a protonated molecular ion at \(m/z\) 337 suggesting hydroxylation of zoniporide. The fragment ions in the mass spectrum of the metabolite were similar to those observed in the mass spectrum of metabolite M1 and M7 (Table 6). The difference in the retention time of M10 from M1 and M7 suggested that M10 was an isomer of these two metabolites however the exact position of the hydroxyl group could not be determined from the mass spectrum. Since the oxidative metabolite was lacking in humans no further attempt to determine the position of hydroxylation in the metabolite.
In Vitro Metabolism Studies.

In order to identify the enzymes responsible for the formation of M1, preliminary experiments were performed by incubating zoniporide with human liver S9 fraction at concentrations of 10 µM in the presence and absence of NADPH (Figure 9A and B). The presence of M1 in the incubations that lacked NADPH suggested that its formation was catalyzed by a non-P450 enzyme. Further, inhibition of M1 in the presence of raloxifene, an aldehyde oxidase inhibitor, to the incubation mixture, indicated that aldehyde oxidase was responsible for its formation (Figure 9C). Only trace amounts of M3 were detected in the mass spectral analysis of the above incubation mixture (data not shown) suggesting that hydrolysis constituted a minor pathway at least in incubations with liver S9 fractions.

Based on the above information, enzyme kinetics studies for formation of M1 by aldehyde oxidase were performed using pooled human liver cytosol and the kinetic parameters were determined. Analysis of the relationship between reaction velocity and substrate concentration revealed substrate inhibition kinetics with kinetic parameters of \( K_M = 3.4 \pm 0.2 \, \mu\text{M}, \ V_{\text{max}} = 74 \pm 2 \, \text{pmol/min/mg protein}, \) and a \( K_s \) value of 152 µM (Figure 10A). A detailed assessment of the molybdenum hydrolases (aldehyde oxidase or xanthine oxidase) was also conducted by determining the IC\(_{50}\) for the inhibition of M1 formation was conducted using specific inhibitors of aldehyde oxidase (raloxifene and menadione) and xanthine oxidase (allopurinol). Raloxifene potently inhibited the reaction (IC\(_{50} = 0.012 \pm 0.002\)), and menadione also inhibited the reaction (9.8 ± 1.2 µM), further confirming the involvement of aldehyde oxidase in M1 formation (Figure 10B). Allopurinol did not inhibit the reaction (IC\(_{50} > 1 \, \text{mM}\)), indicating that xanthine oxidase did not metabolize zoniporide.
In the present study, the routes of elimination, metabolism and the excretion mass balance of zoniporide in humans were investigated following an intravenous infusion of $[^{14}C]$zoniporide. A dose of 80 mg was administered in this study which was approximately equal to the anticipated clinical dose to be used in the phase III program. The comparison of metabolite profiles of a drug candidate in animals and humans is essential to ensure that animal species used in toxicological evaluations are appropriate models of humans and to confirm that all human circulating metabolites are covered in these species (FDA, 2008). The metabolic profile in humans, rat and dog were compared in order to assess if all metabolites observed in human matrices were detected in these two toxicology species. The doses used in the preclinical species were equivalent to the dose in the toxicology studies where minimum adverse effects were observed.

Following IV administration, majority of the radioactivity was excreted in feces suggesting that biliary excretion was the principal route of elimination of zoniporide related material in the humans. Metabolism was the primary route of clearance for zoniporide in humans since only 18% of the dose was excreted unchanged in the urine and feces. Also, the majority of the total circulating radioactivity comprised of metabolites since the exposure of unchanged zoniporide accounted for ~28% of exposure of total circulating radioactivity in plasma. 2-Oxozoniporide (M1), which was formed via oxidation of the quinoline ring of zoniporide, was the primary circulating and excretory metabolite in humans. Its exposure was 2 fold greater than unchanged zoniporide in plasma and approximately 52% of the dose was excreted as M1 in the urine.
and feces. Metabolite M1 was also pharmacologically active against the NHE-1 receptor. However, the activity was ~3 fold less than the parent compound. Since the systemic exposure of M1 was greater than the parent drug and the protein binding was approximately the same as zoniporide, the possibility of the metabolite exerting pharmacological activity in addition to the parent in vivo could not be ruled out. Given that M1 was the major circulating metabolite in humans and was pharmacologically active, in vitro studies using human liver subcellular fractions were performed to identify the enzymes responsible for the formation of M1. Further, this was important in light of the fact of inhibition of the enzyme responsible for the conversion zoniporide to M1 would increase the AUC of the parent drug significantly and would also possibly affect the efficacy of the compound. Phenotyping studies revealed that formation of M1 was primarily catalyzed by aldehyde oxidase (Cl_{int} 22 μl/min/mg cytosolic protein). Aldehyde oxidase is a cytosolic molybdo-flavoenzyme that is expressed predominantly in the liver, lung and kidney and plays a major role in the oxidation of aldehydes and nitrogen containing heterocyclic compounds (Kitamura et. al., 2006). Some heteroaromatic compounds of pharmacological and toxicological importance that are metabolized by aldehyde oxidase include carbezaran (Kaye et. al. 1985), famciclovir (Rashidi, et. al. 1997), methotrexate (Jordan et. al. 1999), zaleplon (Lake et. al. 2002), brimonidine (Acheampong, et. al. 1996), N-[(2’-diethylamino)ethyl]acridine-4-carboximide (Schofield, et. al. 2000). Thus it is not surprising that zoniporide was a substrate of aldehyde oxidase.

Potential drug-drug interactions due to inhibition of aldehyde oxidase have not been established. While cytochrome P450 enzymes have been and continue to be a major
focus of drug interactions, alterations in the activities of other drug-metabolizing enzymes can also be an underlying mechanism of drug-drug interactions. Only one clinically relevant drug-drug interaction between cimetidine and zaleplon has been ascribed to inhibition aldehyde oxidase in the literature so far (Renwick, et. al. 2002). Since several drugs have been demonstrated to be human aldehyde oxidase inhibitors in vitro (Obach et. al. 2004, Obach 2004) it is possible that these drugs could potentially increase the levels of zoniporide in humans. No such drug-drug interactions studies have been conducted and it remains to be determined whether aldehyde oxidase inhibitors identified using in vitro methods could potentially cause a more profound interaction of clinical significance in vivo.

Metabolites M3 and M2, the carboxylic acid analogs of zoniporide and quinolone metabolite (M1), were also observed in humans. This suggested that the hydrolytic cleavage was another pathway by which zoniporide was metabolized. Although M3 accounted for ~20% of parent drug in humans, M2 was found only in the excreta in amounts of ~17% of the dose. The exact metabolic pathway for the formation of M2 was difficult to discern since the metabolite can be formed via two parallel pathways (Figure 8). One pathway could involve hydrolysis of M1 to M2. Alternatively, zoniporide could undergo hydrolysis to M3 which could subsequently undergo an aldehyde oxidase mediated or a P450 mediated oxidation of the quinoline ring to yield M2. Since M2 was not detected in the urine or the plasma of humans, it is possible that the formation of this metabolite in vivo was possibly mediated by microflora in the GI tract.
The metabolism and excretion studies using [\(^{14}\text{C}\)]zoniporide in the rat and dog suggested that the route of excretion of zoniporide in these two species was similar to that in humans and majority of the dose was also eliminated into the feces via the bile. In addition, like humans, zoniporide metabolism was the primary route of clearance in the rat and dog. Only 40 and 37% of the total dose constituted unchanged zoniporide in the rat and dog, respectively. However, comparison of plasma exposure of unchanged zoniporide and total radioactivity in rat and dog revealed that even though circulating radioactivity comprised of metabolites in rats just like humans, unchanged zoniporide was the primary constituent of total radioactivity in dogs.

Analysis of the metabolic profile of zoniporide in the rat revealed that both the human circulating metabolites (M1 and M3), were present in the rat in addition to several other metabolites. Although dogs metabolized zoniporide extensively, in contrast to humans and rats, this species showed differences in its metabolic profile. Metabolite M1, which was a major metabolite in the rats and humans, was not detected in the dogs while M3 was a minor circulating metabolite (6% of the parent) but present in ~9% of the dose in the excreta. The absence of M1 in the dog was not surprising given that dogs lack aldehyde oxidase activity (Kitamura et. al., 2006). Even though oxidation was a primary pathway of metabolism in dogs, the major metabolites observed were the dihydrowiol (M8) and hydroxyzoniporide (M10). Since the other oxidative metabolites detected in the rat and dog were absent in humans no further consideration was given to these metabolites with respect to potential safety implications etc.

An assessment of the coverage of major circulating metabolites in humans was made in this study by comparing estimated exposure levels of M1 and M3 in humans
with those in preclinical species. Assessment was done only in the rat since the dog did not produce M1 metabolite and M3 was produced in <10% parent in vivo. Since levels of M3 in rat exceeded those estimated in humans by 1.7 to 2.0 fold (Table 4) the exposure to this human metabolite was considered covered in the rat. The estimated exposure of M1 in humans on the other hand was comparable to that estimated in rats as depicted in Tables 4 and 5A. Since zoniporide was administered intravenously to humans and the preclinical species, the coverage of metabolite M1 in the preclinical species was also ascertained by estimating the amount of metabolite excreted in the rat and human. This estimation indicated that the amount of M1 to which the rat was exposed was 5.9 fold greater relative to humans. Further, M1 did not have any structural alerts that would pose a risk to humans. Thus, M1 was also considered to be adequately evaluated in the toxicology species and no further assay validation and toxicological evaluation of this metabolite was undertaken in nonclinical safety species. Metabolite M2 was detected only in human feces and was not present in rat and dog, which indicated that this metabolite was unique to the humans. However, since M2 was not present in circulation; was pharmacologically inactive and did not have a functionality that could potentially be a toxicophore, it was not considered for further safety evaluation.

In summary, this study demonstrates the primary routes of excretion and metabolism of zoniporide in humans, rats and dogs. Further the study also demonstrates the strategy that was used to assess if the further toxicological evaluation of the human circulating metabolites was necessary in the preclinical species. Zoniporide is among the few compounds that is almost exclusively metabolized by aldehyde oxidase in humans (with about 3/4th of its clearance mediated by this enzyme) and hence can be used as a
model substrate for this enzyme. Studies are in progress to assess the species differences in the metabolism of aldehyde oxidase and understand the structure metabolism relationship of the compound.
Acknowledgements:

The authors would like to thank the radiochemistry group in Groton for synthesizing $[^{14}\text{C}]$zoniporide.
References


Footnotes

Current Address:

1Genentech, San Francisco, CA.

2Arena Pharmaceuticals, San Diego CA;
Figure Legends

Figure 1  Structure of $[^{14}\text{C}]$zoniporide.
Asterisk indicates the position of the radiolabel.

Figure 2  Mean urine and fecal cumulative recovery of total radioactivity versus time profile in (A) healthy male volunteers ($n = 4$), (B) Sprague Dawley rats ($n = 6$), (C) Beagle dogs ($n = 4$) following intravenous administration of $[^{14}\text{C}]$zoniporide.
The errors bars indicate standard deviation.

Healthy male volunteers were administered $[^{14}\text{C}]$zoniporide by intravenous infusion for 1 h at a dose of 80 mg (100 µCi) and the excreta was collected over 144 h. The rats were administered a bolus of $[^{14}\text{C}]$zoniporide at a dose of 10 mg/kg (50 µCi) and the excreta was collected over 168 h. The dogs were administered a bolus of $[^{14}\text{C}]$zoniporide at a dose of 3mg/kg (150 µCi) and the excreta was collected over 168 h.

Figure 3  Mean plasma concentration-time profiles for total radioactivity and zoniporide following intravenous administration of $[^{14}\text{C}]$zoniporide to (A) healthy male volunteers ($n = 4$); (B) Sprague Dawley rats ($n = 6$) and (C) Beagle dogs ($n = 4$).
The errors bars indicate standard deviation.

Healthy male volunteers were administered $[^{14}\text{C}]$zoniporide by intravenous infusion for 1 h at a dose of 80 mg (100 µCi). The rats were administered a bolus of $[^{14}\text{C}]$zoniporide at
a dose of 10 mg/kg (50 μCi). The dogs were administered a bolus of [14C]zoniporide at a
dose of 3 mg/kg (150 μCi).

Figure 4 Representative HPLC radiochromatogram of a pooled urine (A) and feces
(B) following intravenous infusion of [14C]zoniporide for 1 h at a dose of
80 mg (100 μCi) to healthy male volunteers.

Figure 5 Representative HPLC radiochromatogram of a pooled urine (A) and feces
(B) following intravenous administration of [14C]zoniporide at a dose of
10 mg/kg (50 μCi) to Sprague Dawley rats.

Figure 6 Representative HPLC radiochromatogram of urine (A) and feces (B)
following intravenous administration of [14C]zoniporide at a dose of 3
mg/kg (150 μCi) to Beagle dogs.

Figure 7 HPLC-radiochromatogram of pooled plasma of healthy male volunteers
following intravenous infusion of 80 mg [14C]zoniporide for 1 h (A), and
Sprague Dawley rats (B) and Beagle dogs (C) following intravenous
administration of [14C]zoniporide at a dose of 10 and 3 mg/kg,
respectively.
Figure 8  Proposed metabolic scheme of zoniporide in healthy male volunteers, Sprague Dawley rats and Beagle dogs following intravenous administration.

Figure 9  Metabolic profile of zoniporide (10 μM) following incubation with human liver S-9 fractions in the A) presence of NADPH; B) absence of NADPH and C) in the presence of raloxifene, an inhibitor of aldehyde oxidase.

Figure 10  Enzyme kinetics for the formation of 2-oxozoniporide (M1), from zoniporide in pooled human liver cytosol (A) and inhibition of this reaction by raloxifene, menadione, and allopurinol (B).

The enzyme kinetics experiments were done in triplicate and the inhibition studies were done in duplicate. The errors bars in panel A indicate standard deviation.
Table 1. Percent recovery of the dose in healthy male volunteers, Sprague Dawley rats and Beagle dogs after intravenous administration of $[^{14}\text{C}]$zoniporide.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Urine (%)</th>
<th>Feces (%)</th>
<th>Total (%)</th>
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<td>0-24</td>
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</tr>
<tr>
<td>0-144</td>
<td>27</td>
<td>61</td>
<td>88</td>
</tr>
<tr>
<td>0-168</td>
<td>27</td>
<td>62</td>
<td>89</td>
</tr>
</tbody>
</table>

$[^{14}\text{C}]$Zoniporide was administered by intravenously to humans at a dose of 80 mg and to Sprague Dawley rats and Beagle dogs at a dose of 10 and 3 mg/kg, respectively.
Table 2. Mean pharmacokinetic parameters of total circulating radioactivity and zoniporide in healthy male volunteers, Sprague Dawley rats and Beagle dogs following intravenous administration of [\(^{14}\text{C}\)]zoniporide.

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameters</th>
<th>Units</th>
<th>Healthy Male Volunteers</th>
<th>Sprague Dawley Rats</th>
<th>Beagle Dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Radioactivity</td>
<td>Zoniporide</td>
<td>Radioactivity</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>1.0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Cmax (ng/mL)</td>
<td>899</td>
<td>237</td>
<td>590</td>
<td>44.2</td>
</tr>
<tr>
<td>AUC(_{0-\text{tlast}}) (ng-h/mL)</td>
<td>2925</td>
<td>223</td>
<td>833</td>
<td>33.4</td>
</tr>
<tr>
<td>AUC(_{0-\infty}) (ng-h/mL)</td>
<td>3250</td>
<td>122</td>
<td>839</td>
<td>34.2</td>
</tr>
<tr>
<td>T(_{1/2}) (h)</td>
<td>6.7</td>
<td>3.3</td>
<td>2.0</td>
<td>0.4</td>
</tr>
<tr>
<td>Cl (mL/min/kg)</td>
<td>21.0</td>
<td>1.2</td>
<td>113</td>
<td>22</td>
</tr>
<tr>
<td>Vss (L/Kg)</td>
<td>1.7</td>
<td>0.4</td>
<td>2.2</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Plasma concentrations for total radioactivity were expressed in ng-equiv./mL.

AUC\(_{0-\text{tlast}}\) and AUC\(_{0-\infty}\) for radioactivity was expressed in ng-equiv.-h/mL.

Healthy male volunteers were administered [\(^{14}\text{C}\)]zoniporide by intravenous infusion for 1 h at a dose of 80 mg (100 µCi). The Sprague Dawley rats and Beagle dogs were administered a bolus of [\(^{14}\text{C}\)]zoniporide at a dose of 10 mg/kg (50 µCi) of and 3mg/kg (150 µCi).
Table 3. Percentage of urinary and fecal metabolites of zoniporide following intravenous administration of \([^{14}\text{C}]\)zoniporide to healthy male volunteers, Sprague Dawley rats and Beagle dogs.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>% of Dose Excreted</th>
<th>Healthy Male Volunteers</th>
<th>Sprague Dawley Rats</th>
<th>Beagle Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>Feces</td>
<td>Total</td>
</tr>
<tr>
<td>M1</td>
<td>16</td>
<td>36</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>17</td>
<td>17</td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>M3</td>
<td>0.8</td>
<td></td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>M4</td>
<td>2.1</td>
<td></td>
<td></td>
<td>2.1</td>
</tr>
<tr>
<td>M5</td>
<td>0.76</td>
<td>1.3</td>
<td>2.1</td>
<td>1.2</td>
</tr>
<tr>
<td>M6</td>
<td>1.4</td>
<td></td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td>M7</td>
<td></td>
<td>5.9</td>
<td></td>
<td>5.9</td>
</tr>
<tr>
<td>M8</td>
<td></td>
<td>1.7</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>M9</td>
<td></td>
<td></td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>M10</td>
<td></td>
<td>0.29</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Zoniporide</td>
<td>17</td>
<td>1.0</td>
<td>18</td>
<td>28</td>
</tr>
</tbody>
</table>

Healthy male volunteers were administered \([^{14}\text{C}]\)zoniporide by intravenous infusion for 1 h at a dose of 80 mg (100 µCi). The rats were administered a bolus of \([^{14}\text{C}]\)zoniporide at a dose of 10 mg/kg (50 µCi). The dogs were administered a bolus of \([^{14}\text{C}]\)zoniporide at a dose of 3mg/kg (150 µCi).
Table 4. Percentage of circulating metabolites of zoniporide and their estimated exposure in healthy male volunteers, Sprague Dawley rats and Beagle dogs following intravenous administration of $[^{14}C]$zoniporide.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Human</th>
<th>Rat</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% circulating radioactivity</td>
<td>Estimated AUC$<em>{(0-t</em>{last})}$ (ng-equiv.-h/mL)</td>
<td>% circulating radioactivity</td>
</tr>
<tr>
<td>M1</td>
<td>60</td>
<td>1755</td>
<td>46</td>
</tr>
<tr>
<td>M3</td>
<td>6.4</td>
<td>187</td>
<td>9.0</td>
</tr>
<tr>
<td>M4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M8</td>
<td>8.5</td>
<td>303</td>
<td>5.4</td>
</tr>
<tr>
<td>M9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>zoniporide</td>
<td>30</td>
<td>878</td>
<td>36</td>
</tr>
</tbody>
</table>

Healthy male volunteers were administered $[^{14}C]$zoniporide by intravenous infusion for 1 h at a dose of 80 mg (100 µCi). The rats were administered a bolus of $[^{14}C]$zoniporide at a dose of 10 mg/kg (50 µCi). The dogs were administered a bolus of $[^{14}C]$zoniporide at a dose of 3 mg/kg (150 µCi).

The estimated exposures were determined by multiplying % circulating radioactivity$_{metabolite}$ and AUC$_{(0-t_{last})}$ radioactivity.$^{-1}$

The AUC$_{(0-t_{last})}$radioactivity is depicted in Table 2.
Table 5A. Estimation of exposure of metabolites M1 and M3 in rat plasma.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Human</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M/P Ratio</td>
<td>Estimated Exposure</td>
</tr>
<tr>
<td>M1</td>
<td>2</td>
<td>1666</td>
</tr>
<tr>
<td>M3</td>
<td>0.21</td>
<td>178</td>
</tr>
</tbody>
</table>

Metabolite to parent ratio was determined from the abundance of the metabolite (% of total circulating radioactivity) and parent (% of total circulating radioactivity) in the plasma.

Estimated exposure of Metabolite = (Metabolite/Parent ratio) • AUC_{zoniporide}

AUC of zoniporide was determined following quantitation of the parent by a bioanalytical assay.
Table 5B. Estimation of total body burden of M1 from excretion data in humans and rats.

<table>
<thead>
<tr>
<th></th>
<th>Dose (mg)</th>
<th>Dose (mg/kg)</th>
<th>% Dose of M1</th>
<th>Amount (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humans</td>
<td>80</td>
<td>1.14</td>
<td>52</td>
<td>594</td>
</tr>
<tr>
<td>Rats</td>
<td>NA</td>
<td>10</td>
<td>35</td>
<td>3500</td>
</tr>
</tbody>
</table>

NA = Not available.

Amount of M1 in the excreta was determined using equation 3.
Table 6. Mass Spectral fragmentation and structures of zoniporide and proposed metabolites.

<table>
<thead>
<tr>
<th>Structure</th>
<th>[M+H]^+</th>
<th>Fragment Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zoniporide</td>
<td>321, 262, 234, 220</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>337, 278, 250, 236</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>296, 278, 250</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>280, 262, 234</td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td>337, 320, 278, 261, 250</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2

A

% of Dose Excreted

Time (h)

B

% of Dose Excreted

Time (h)

C

% of Dose Excreted

Time (h)
Figure 3
Figure 4
Figure 5
Figure 7

A

M1

Zoniporide

M3

B

M1

Zoniporide

M6

M3

C

M9

M8

M4

M10

M3

Radioactivity (CPM)

Time (min)

Radioactivity (CPM)

Time (min)

Radioactivity (CPM)

Time (min)
Figure 9
Figure 10