Assessment of Exposure of Metabolites in Preclinical Species and Humans at Steady State from the Single-Dose Radiolabeled Absorption, Distribution, Metabolism, and Excretion Studies: A Case Study

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ABSTRACT:

The exposure of a drug candidate and its metabolites in humans and preclinical species during drug development needs to be determined to ensure that the safety of drug-related components in humans is adequately assessed in the standard toxicology studies. The in vivo radiolabeled studies in preclinical species and human volunteers provide the total fate of the drug-derived radioactivity including the relative abundance of metabolites. Here, we describe how the single-dose radiolabeled human studies could provide the exposure of circulating metabolites at steady state using a case study of an extensively metabolized drug, lixivaptan. After an oral dose of $[^{14}C]$lixivaptan to humans, a total of nine metabolites were detected in the systemic circulation; eight of them exceeded 10% of the parent exposure (2–41% of total radioactivity). The plasma samples were profiled for all subjects at each time point by high-performance liquid chromatography, and metabolites were quantified using a radioactive detector. On the basis of single-dose area under the concentration-time curve (AUC) values, exposure of six human metabolites was greater at least in one preclinical species used in toxicology evaluation. On the basis of the $t_{1/2}$ of lixivaptan and two major metabolites from a single dose in humans, their AUC and $C_{\text{max}}$ values were simulated at the steady state. The simulated exposure ($C_{\text{max}}$ and AUC) values of parent drug and the two most abundant metabolites were similar to those from a 7-day clinical study obtained using a validated liquid chromatography-mass spectrometry assay, suggesting that a well designed single-dose radiolabeled human study can help in addressing the metabolites in safety testing-related issues.

Introduction

Understanding the metabolic fate of a new chemical entity (NME) in preclinical species and humans is an integral part of its comprehensive safety evaluation. For the vast majority of NMEs, information on their metabolic fate and exposure of metabolites is required for regulatory approval. Metabolic pathways of the NMEs in animals, used for safety evaluation studies, are required to ensure that the selected animal species are exposed to all major metabolites formed in humans (Baillie et al., 2002). In 2005, the U.S. Food and Drug Administration issued a draft guidance that provided a general outline regarding when and how to evaluate safety of major and unique human metabolites in safety testing (MIST), which was then finalized in 2008 (U.S. Food and Drug Administration, Guidance for Industry: Safety Testing of Drug Metabolites, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm079266.pdf). The guidance recommends that human circulating metabolites that exceed 10% of parent exposure at steady state should have equal or greater exposure at least in one animal species used for safety assessment. If this is not achieved, then additional toxicological testing on metabolites may be required to achieve the recommended exposure to the metabolite(s). This Food and Drug Administration guidance sparked a debate among the regulators and the industry and was critically evaluated in several publications (Davis-Bruno and Attrakchi, 2006; Prueksaritanont et al., 2006; Smith and Obach, 2006, 2009; Leclercq et al., 2009; Robison and Jacobs, 2009; Smith et al., 2009). The more recent 2009 International Conference on Harmonization guidance [Non-Clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals M3 (R2), http://www.emea.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002720.pdf] recommends that nonclinical characterization of human metabolite(s) is only warranted when a metabolite(s) had an exposure greater than 10% of total drug-related exposure and at significantly higher levels in humans than the maximum exposure in the toxicity studies.

To mitigate this risk of human metabolites in safety testing, several analytical approaches based on UV, MS, and/or NMR responses have been described to compare the exposure of drug metabolites in plasma from preclinical species and humans without the synthesis of authen-

ABBREVIATIONS: NME, new chemical entity; MIST, metabolites in safety testing; MS, mass spectrometry; AUC, area under the concentration-time curve; HPLC, high-performance liquid chromatography; BDC, bile duct-cannulated; LC, liquid chromatography; MS/MS, tandem mass spectrometry; PK, pharmacokinetic; ADME, absorption, distribution, metabolism, and excretion; AME, absorption, metabolism, and excretion.
tic standards (Vishwanathan et al., 2009; Gao et al., 2010; Ma et al., 2010). These approaches are useful to compare the metabolic profiles between humans and preclinical species but may not provide their exposure because establishing a correlation between an MS signal for a metabolite and its actual amount is difficult without a synthetic standard (Ramanathan et al., 2007; Penner et al., 2010). In addition, for an extensively metabolized drug, there may be several metabolites greater than 10% of total AUC, therefore, requiring considerable efforts and investment in the synthesis of multiple metabolite standards and development of a rigorous quantitative bioanalytical assay(s) for multiple analytes (Humphreys and Unger, 2006). In vivo studies using radiolabeled drug provide definitive data on the metabolic profiles and relative abundance of metabolites in plasma and urine (Penner et al., 2009, 2012). We describe here plasma profiling strategies to assess the exposure of circulating metabolites in humans of an extensively metabolized drug candidate, lixivaptan, at steady state after administration of a single dose of the radiolabeled drug.

Lixivaptan (VPA-985; 5-fluoro-2-methyl-N-[4-(5H-pyrrolo[2,1-C][1,4]-benzodiazepin-10(1H)-ylcarbonyl]-3-chlorophenyl]-benzamide) is a potent, selective, orally active, nonpeptide competitive vasopressin V2 receptor antagonist (Albright et al., 1998; Chan et al., 1998; Ashwell et al., 2000; Zmily et al., 2011). It is currently being developed for the treatment of hyponatremia associated with syndrome of inappropriate antidiuretic hormone secretion and heart failure. In clinical studies, lixivaptan appears to be safe and effective in correcting hyponatremia as well as inducing a negative fluid balance without activation of the neurohumoral system, suggesting that it has therapeutic potential in managing water retention in patients with liver cirrhosis with ascites (Decaux, 2001; Wong et al., 2003; Abraham et al., 2006).

Preclinical pharmacokinetic studies suggested that lixivaptan is well absorbed. Based on its clearance values, it is likely that first-pass hepatic metabolism is largely responsible for the low bioavailability in rats (7–25%) and dogs (23%). In humans, it is rapidly absorbed, and Cmax was achieved within 1 h after administration. The elimination half-life was 9.0 h after a 100-mg oral dose (Muralidharan et al., 1999; Guyader et al., 2002). MS analysis of plasma from phase I studies indicated that lixivaptan is extensively metabolized, and several metabolites showed higher MS response than the parent compound. The current study was performed to compare the metabolism and excretion of lixivaptan in humans and to assess the coverage of all human circulating metabolites in toxicity species, rats and dogs. [14C]Lixivaptan was orally administered to young healthy male volunteers, Sprague-Dawley rats, and dogs, and the metabolic profiles were obtained in plasma. The exposures (Cmax and AUC) of lixivaptan and its most abundant metabolites from a single dose in humans were simulated at the steady state and compared with those obtained from a 7-day clinical study.

Materials and Methods

General. Commerically obtained chemicals and solvents were of HPLC or analytical grade. The Zorbax Rx-C18 (5 μm; 3.0 × 250 mm) analytical column was purchased from MAC-MOD Analytical Inc. (Chadds Ford, PA). Ultima Gold XR, Carbo-Sorb, and Permafluor E analytical column was purchased from MAC-MOD Analytical Inc. (Chadds Ford, PA); Ultima Gold XR, Carbo-Sorb, and Permafluor E were obtained from Sigma-Aldrich (St. Louis, MO). Radiolabeled F68 was obtained from BASF Inc. (Mt. Olive, NJ).

Radiolabeled Drug and Reference Compounds. [14C]Lixivaptan (5-fluoro-2-methyl-N-[4-(5H-pyrrolo[2,1-C][1,4]-benzodiazepin-10(1H)-ylcarbonyl]-3-chlorophenyl]-benzamide; specific activity 30–38 μCi/mg) (Fig. 1), uniformly radiolabeled at the carbonyl between the fluorobenzamide and the chlorophenyl ring was synthesized at Wyeth (Princeton, NJ). The radiochemical purity of radiolabeled material was ≥97%, as determined by HPLC using an in-line radioactivity detector, for all studies. The metabolites M3 (WAY-138451; N-[3-chloro-4-(3-oxo-5,10,11,15-tetrahydro-3H-benz[b][1,4]diazepine-10-carbonyl]-phenyl]-5-fluoro-2-methyl-benzamide), M4 (WAY-137930; N-[3-chloro-4-(3-oxo-2,3-dihydro-1H-quinazolin-2-yl]-benzoylamino)-benzyl]-3-(pyrrol-2-yl)-1H-pyrrole-2-carboxylic acid), and M6 (WAY-138758; 2-(2-chloro-4-fluoro-2-methyl-1H-benzimidazol-2-yl)-5-fluoro-2-methyl-benzamide), M5 (WAY-141624; 1-(2-[2-chloro-4-(5-fluoro-2-methyl-benzoylamino)benzoylamino]-benzy1)-1H-pyrrole-2-carboxylic acid), and M6 (WAY-138758; 2-(2-chloro-4-fluoro-2-methyl-1H-benzimidazol-2-yl)-5-fluoro-2-methyl-benzamide) were synthesized as described earlier (Ashwell et al., 2000; Molinari et al., 2007).

Study Design, Dosing, and Sample Collection in Humans. Six healthy male subjects between the ages of 18 and 45 years participated in the study. All subjects provided written, informed consent before participation in the study. The study protocol, consent documents, consent procedures, and subject recruitment procedures were approved by the independent institutional review board. The study was conducted in compliance with the International Conference on Harmonization Good Clinical Practices guidelines, the ethical principles that have their origin in the Declaration of Helsinki, and the U.S. Food and Drug Administration regulations for informed consent and protection of patient rights. The subjects entered the clinical research facility (International Medical Technical Consultants, Inc., Lenexa, KS) 12 h before dosing and remained there for up to 168 h after dosing under continuous medical observation. A single oral dose of 100 mg (200 μCi) of [14C]lixivaptan (two 50-mg capsules) was administered with 250 ml of water. The subjects abstained from food and fluids (except water) for at least 10 h before and 4 h after dose administration. The drug was administered in an open fashion as a single oral dose in the morning. A standard meal was provided 4 h later.

Each subject emptied his bladder and provided a 50-ml sample of urine immediately before dosing, which was frozen at or below −20°C. After dosing, urine samples were collected at 0 to 4, 4 to 8, 8 to 12, and 12 to 24 h and than at 24-h intervals for up to 168 h. All of the urine collected during each time period was mixed, and the total volume was recorded. Fecal samples were collected as passed for up to 168 h. Venous blood samples (≈10 ml) for lixivaptan concentrations and total radioactivity were collected from each subject in glass vacuum blood collection tubes before dosing (0) and at 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36, 48, 96, 120, and 144 h postdose. For the analysis of lixivaptan metabolites, venous blood samples (≈50 ml) were collected from each subject as described above at 0.5, 2, 4, 8, 12, 24, and 36 h postdose. All blood samples were centrifuged, and plasma samples were collected in separated clean tubes. All these samples were stored at or below −20°C until analyzed.

Animals, Dosing, and Sample Collection. Intact and bile duct-cannulated (BDC) Sprague-Dawley rats (250–350 g) were obtained from Charles River Laboratories (Kingston, NY). Beagle dogs (10–12 kg) were obtained from Marshall Breeding Laboratories (North Rose, NY). Animals were quarantined for approximately 4 days before dose administration and maintained on a 12-h light/dark cycle. All animals were housed in individual, suspended, stainless steel, wire mesh cages during acclimation. During the test period, animals were housed as paired for sample collection. Rats designated for collection of bile were housed in individual, suspended, stainless steel cages until the bile canulae were exteriorized and subsequently in Nalgene metabolism cages designed for the separation and collection of urine and feces and the collection of bile. The animals were fasted 18 h before administration of the dose and were fed 4 h after the dose. The animals were provided water ad libitum. The drinking water for BDC rats was supplemented with 1% w/v bile salts, 0.9% w/v NaCl, and 0.05% w/v KCl to replenish electrolytes lost during the 48-h collection of bile. All animal studies were conducted in a research facility accredited by the American Association for the Accreditation of Laboratory Animal Care.

Rats. Mass balance and routes of excretion of lixivaptan were investigated in Sprague-Dawley rats after oral administration of [14C]lixivaptan. In addition, pharmacokinetics of radioactivity and lixivaptan were obtained after oral administration of [14C]lixivaptan to intact rats. One group of male BDC (group 1; n = 5) and another group of intact (group 2; three per gender) rats received a single oral dose of [14C]lixivaptan (10 mg/kg, 10 mCi) as a homogeneous suspension in deionized water and Pluronic F68 by gavage. For pharmacokinetics of radioactivity and lixivaptan, a third group of jugular vein-cannulated...
rats (group 3, \( n = 5 \) per gender) received a single oral as described above. For identification of circulating metabolites, a fourth group of animals (group 4, \( n = 9 \) per gender) received a single oral dose of [\(^{14}\text{C}\)]lixivaptan (10 mg/kg, 10 ml/kg) as described above. The specific activity of both formulations was \( 29.8 \mu\text{Ci/mg} \).

Bile samples were collected from the bile duct-cannulated rats (group 1) at intervals of 0 to 4, 4 to 8, 8 to 24, and 24 to 48 h. The bile was collected on wet ice. Urine and feces were collected from intact rats (group 2) at 0 to 8 and 8 to 24 h and at every 24-h interval for 7 days. The first fecal sample was collected at 24 h postdose. For pharmacokinetics, blood samples (1 ml) were collected into tubes containing K\(_2\)EDTA anticoagulant via the jugular vein from rats (group 3) at 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48, 72, 120, and 168 h postdose. After removal of each blood sample starting with the 1-h sample, animals received 1 ml of donor blood to maintain blood volume and hematocrit. Blood samples (\( 10 \) ml) from three rats per time point per gender (group 4) were collected by cardiac puncture under isoflurane anesthesia in 10-ml K\(_2\)EDTA Vacutainer tubes at 1, 8, and 24 h for metabolite identification. Animals were euthanized by CO\(_2\) inhalation after each blood sampling time. Blood was centrifuged for 15 min at 4°C, and the resulting plasma was removed. All of the biological samples were stored at \( -20°C \) until analysis.

**Dogs.** One group of four male dogs received an oral (10 mg/kg, 2 ml/kg) dose of [\(^{14}\text{C}\)]lixivaptan by gavage followed by a 10-ml water rinse of the dosing apparatus. The oral dosing suspension (5.0 mg/ml) was prepared by mixing [\(^{14}\text{C}\)]lixivaptan and Pluronic F68 in deionized water. The specific activity of the dosing formulation was 1.04 \( \mu\text{Ci/mg} \).

For pharmacokinetics of total radioactivity and lixivaptan, blood samples (5 ml) were collected into tubes containing K\(_2\)EDTA anticoagulant via the jugular vein from dogs at 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48, 72, 120, and 168 h postdose. An additional blood sample (10 ml) was collected into tubes containing K\(_2\)EDTA anticoagulant via the jugular vein of each orally dosed dog at 2, 8, and 24 h for metabolite identification. The blood was centrifuged for 15 min at 4°C, and the resulting plasma was removed. Urine and fecal samples were collected frozen over dry ice at 0 to 8 and 8 to 24 h and at every 24-h interval for 7 days. The first fecal sample was collected at 24 h postdose. All of the biological samples were stored at \( -20°C \) until analysis.

**Determination of Radioactivity.** The radioactivity in urine, bile, feces, and plasma was determined by liquid scintillation counting. Duplicate aliquots of urine, bile, or plasma were mixed with scintillation cocktail for radioanalysis. The weight of each fecal sample was determined, and the feces were homogenized with water (20% w/w, feces/water). The total weight of the homogenate was recorded. After homogenization, duplicate aliquots were transferred into tared cones and pads, weighed, and dried overnight at ambient temperature before combustion in a model 307-D Tri-Carb Sample Oxidizer (Canberra Industries, Meriden, CT) equipped with an Oximate-80 Robotic Automatic Sampler (PerkinElmer Life and Analytical Sciences). Radioactivity in the combustion products was determined by trapping the liberated CO\(_2\) in CarboSorb followed by liquid scintillation counting using Permafluor E+ as a scintillation cocktail. Combustion efficiency was determined daily before the combustion of study samples, using diluted dose formulation or a \( ^{14}\text{C} \) standard. The measured radioactivity content in combusted samples was adjusted using the combustion efficiency values.

Samples collected before dosing were used as controls and counted to obtain a background count rate. Radioactivity less than twice the background value (40 dpm) was considered to be below the limit of determination. The radioactivity in the dose was established as 100% of the total radioactivity. The radioactivity at each sampling time for urine, bile, and feces was defined as the percentage of dose excreted in the respective matrices. The amount of radioactivity in plasma was expressed as nanogram equivalents of parent drug per milliliter and was calculated by using the specific activity of the dose administered.
Extraction of Metabolites from Plasma. Plasma samples were added to 2 volumes of acetonitrile, vortexed, and sonicated (—10 min). The mixtures were centrifuged at 3000 rpm for approximately 10 min, and the supernatants were placed in clean 50-mL Falcon polypropylene conical tubes. The pellets were extracted with an additional 2 volumes of acetonitrile, the mixtures were centrifuged at 3000 rpm for approximately 10 min, and the supernatants were combined. Aliquots (0.1–0.2 mL) were analyzed by liquid scintillation counting. The mean recovery of radioactivity after extraction was determined to be >85.0%. The supernatants were evaporated to dryness under nitrogen in a TurboVap LV evaporator (Caliper Life Sciences, Hopkinton, MA). The residues were then reconstituted in acetonitrile, and an equal amount of water was added. Aliquots (100 μL) were injected onto the HPLC system.

HPLC. The HPLC system consisted of a model 600-MS pump (Waters, Milford, MA) connected to a SpectroMonitor 3200 variable wavelength detector (monitoring 270 nm; LDC/Milton Roy, Riviera Beach, FL), a model 715/1012 oms/cm2; collision offset, 35 eV; and resolution (Q1 and Q3), 0.7 ± 0.2 Da at 50% peak height.

Quantitation of Lixivaptan and Its Metabolites in Plasma. The systemic exposure of lixivaptan and two abundant human metabolites (M5 and M6) was quantitatively determined in human plasma obtained from a phase 1 study after oral administration of a 100-mg dose of lixivaptan daily for 8 days, and blood samples were collected at day 8. Lixivaptan and its two metabolites (M5 and M6) were quantitated in human plasma using the validated LC-MS/MS assay. Deuterated (d1-lixivaptan, d3-M5, and d5-M6) analogs were used as the internal standards. Multiple reaction monitoring transitions for lixivaptan and M5 and M6 were 474.1 → 290.0, 490.1 → 290.0, and 427.1 → 290.0, respectively. The corresponding multiple reaction monitoring transitions for the internal standards of lixivaptan and M3, M5, and M6 were 478.2 → 290.0, 494.1 → 290.0, and 431.1 → 290.0, respectively. The dynamic ranges for all analytes were 5 to 2000 ng/mL in human plasma.

Pharmacokinetic Analysis. Pharmacokinetic parameters were determined using WinNonlin Professional Software (version 5.3; Pharsight, Mountain View, CA). Maximum observed concentrations (Cmax) of lixivaptan or total radioactivity (parent drug equivalents) in plasma were estimated directly from the experimental data, with Tmax defined as the time of first occurrence of Cmax. Terminal phase rate constants (k) were estimated using least-squares regression analysis of the serum concentration-time data obtained during the terminal log-linear phase. Half-life (t1/2) was calculated as 0.693/k. Area under the serum concentration-time curve from time 0 to the last time (t) with a measurable concentration (AUC(0→t)) was estimated using the linear trapezoidal rule. AUC from time t to infinity (AUC(0→∞)) was estimated as Cmax/k where Cmax represents the estimated concentration at time t based on the aforementioned regression analysis. AUC from time 0 to infinity (AUC(0→∞)) was estimated as the sum of AUC(0→t) and AUC(0→∞) values. Cmax and AUC of lixivaptan and its metabolites at steady state were estimated on the basis of AUC(0–24 h) after the first dose and the accumulation factor derived from the elimination half-life t1/2 using the equation R = 1/(1 – exp(−τ × (0.693/t1/2)))). Cmax and AUC of lixivaptan and its metabolites at steady state were estimated by multiplying the value after a single dose by its respective R. The use of this

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**TABLE 1**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Dose</th>
<th>% Radioactive Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Urine</td>
</tr>
<tr>
<td>Human</td>
<td>1</td>
<td>20.5 ± 4.1</td>
</tr>
<tr>
<td>Male rats</td>
<td>10</td>
<td>5.77 ± 0.25</td>
</tr>
<tr>
<td>Female rats</td>
<td>10</td>
<td>7.60 ± 1.4</td>
</tr>
<tr>
<td>Male rats</td>
<td>10</td>
<td>N.D.</td>
</tr>
<tr>
<td>Female rats</td>
<td>10</td>
<td>70.5 ± 10.3</td>
</tr>
<tr>
<td>Male dog</td>
<td>10</td>
<td>7.95 ± 3.4</td>
</tr>
</tbody>
</table>

N.A., not applicable; N.D., not detected.

*Includes cage wash/debris.

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**FIG. 2.** Mean (±S.D.) plasma concentration-time curves of lixivaptan and total radioactivity in humans.
method to estimate steady-state PK parameters is based on the assumption that the pharmacokinetics of lixivaptan or its metabolites are not time-dependent.

Results

Excretion Studies. Humans. After a single oral dose of $^{14}$C]lixivaptan to human subjects, the majority of the radioactivity was excreted in the feces (Table 1), with a mean total of 63.7% of the radioactivity at 168 h postdose. A mean total of 84.2% of the radioactivity was excreted in the urine and feces over a period of 168 h postdose. The mean urinary recovery was 20.5%. The majority of recovered radioactivity ($>95\%$) was excreted within the first 96 h after administration of the radioactive dose in all subjects.

Rats. The radioactive dose was quantitatively recovered in Sprague-Dawley rats after oral administration (99.0% for males and 103.1% for females) after 168 h (Table 1). Most of the radioactivity was eliminated in the feces (91.0% for males and 92.0% for females). Urinary excretion was minor with 5.8% for males and 7.6% for females being recovered after the oral dose.

In bile duct-cannulated rats, the mean cumulative biliary excretion of radioactivity from 0 to 48 h after an oral dose (10 mg/kg) of $^{14}$C]lixivaptan was 65.4 and 70.5% for males and females, respectively (Table 1). Biliary excretion of radioactivity was rapid, with approximately 70 to 80% of the recovered radioactivity being excreted in the first 8 h.

Dogs. The radioactive dose was quantitatively recovered in dogs after an oral dose (96.0%) over a period of 168 h (Table 1). Most of the radioactivity (83.1%) was eliminated in the feces after oral administration. Urinary excretion was minor with 8.0% being recovered after an oral dose (Table 1).

Pharmacokinetics of Unchanged Lixivaptan and Radioactivity in Plasma. Humans. The mean concentration-time profiles of lixivaptan and total radioactivity in plasma of humans after oral administration of $^{14}$C]lixivaptan are graphically depicted in Fig. 2. $^{14}$C]Lixivaptan was rapidly absorbed with a $T_{	ext{max}}$ of radioactivity in plasma of 1.2 h. The individual values for $C_{\text{max}}$ of total radioactivity ranged from 1110 to 2670 ng Eq/ml (mean of 1770 ng Eq/ml). Mean $AUC_{0\text{ to }\infty}$ values were 24,700 and 26,200 ng Eq·h/ml, respectively (Table 2).

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$C_{\text{max}}$ and $AUC$ values for total radioactivity are expressed as nanogram equivalents per milliliter and nanogram equivalents per hour per milliliter, respectively.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Gender</th>
<th>Dose</th>
<th>$T_{\text{max}}$</th>
<th>$C_{\text{max}}$ $^\text{b}$</th>
<th>$t_{1/2}$</th>
<th>$AUC_{0\text{ to }\infty}$ $^\text{b}$</th>
<th>$AUC_{0\text{ to }\infty}$ $^\text{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radioactivity</td>
<td>Human</td>
<td>100 mg</td>
<td>1.2 ± 0.4</td>
<td>1770 ± 590</td>
<td>25.5 ± 7.29</td>
<td>24,700 ± 5860</td>
<td>26,200 ± 6060</td>
</tr>
<tr>
<td>Lixivaptan</td>
<td>Human</td>
<td>100 mg</td>
<td>0.7 ± 0.3</td>
<td>437 ± 120</td>
<td>9.77 ± 3.13</td>
<td>1540 ± 177</td>
<td>1570 ± 177</td>
</tr>
<tr>
<td>Radioactivity</td>
<td>Male rats</td>
<td>10 mg/kg</td>
<td>3.2 ± 0.8</td>
<td>2820 ± 320</td>
<td>36.6 ± 3.6</td>
<td>65,700 ± 18,700</td>
<td>67,100 ± 19,500</td>
</tr>
<tr>
<td>Lixivaptan</td>
<td>Male rats</td>
<td>10 mg/kg</td>
<td>1.4 ± 0.5</td>
<td>1960 ± 220</td>
<td>33.6 ± 4.7</td>
<td>36,700 ± 5000</td>
<td>37,200 ± 5100</td>
</tr>
<tr>
<td>Radioactivity</td>
<td>Female rats</td>
<td>10 mg/kg</td>
<td>0.8 ± 0.6</td>
<td>91 ± 46</td>
<td>1.3 ± 0.2</td>
<td>140 ± 64</td>
<td>219 ± 55</td>
</tr>
<tr>
<td>Lixivaptan</td>
<td>Female rats</td>
<td>10 mg/kg</td>
<td>0.8 ± 0.3</td>
<td>338 ± 80</td>
<td>4.2 ± 3.4</td>
<td>1530 ± 640</td>
<td>1890 ± 840</td>
</tr>
<tr>
<td>Radioactivity</td>
<td>Male dogs</td>
<td>10 mg/kg</td>
<td>1.8 ± 0.8</td>
<td>3570 ± 780</td>
<td>69.7 ± 14.9</td>
<td>82,800 ± 17200</td>
<td>94,900 ± 19,900</td>
</tr>
<tr>
<td>Lixivaptan</td>
<td>Male dogs</td>
<td>10 mg/kg</td>
<td>0.8 ± 0.4</td>
<td>759 ± 218</td>
<td>8.8 ± 2.1</td>
<td>4700 ± 1440</td>
<td>4870 ± 1420</td>
</tr>
</tbody>
</table>

$^a$ $C_{\text{max}}$ and $AUC$ values for total radioactivity are expressed as nanogram equivalents per milliliter and nanogram equivalents per hour per milliliter, respectively.

![Fig. 3. Mean (±S.D.) plasma concentration-time curves of lixivaptan and total radioactivity in rat (A) and dog (B).](image-url)
administration. Mean $\text{AUC}_{0-t}$ and $\text{AUC}_{0-\infty}$ values were 1540 and 1570 ng $\cdot$ h/ml, respectively (Table 2). The mean $t_{1/2}$ was 9.8 h. Plasma concentrations for unchanged lixivaptan were much lower than those for total radioactivity. Mean parameter ratios of unchanged lixivaptan to total plasma radioactivity were 0.25 ($C_{\text{max}}$, and 0.06 ($\text{AUC}_{0-t}$ and $\text{AUC}_{0-\infty}$). These large differences indicate that the majority of the radioactive material in the systemic circulation represents metabolites of lixivaptan and that some of these metabolites are eliminated at slower rates than the parent compound.

**Rats.** The plasma concentration-time curves for radioactivity and lixivaptan in male and female rats after oral administration (10 mg/kg) of [14C]lixivaptan are shown graphically in Fig. 3A. $C_{\text{max}}$ of radioactivity ranged from 2250 to 3130 ng Eq/ml (mean 2820 ng Eq/ml) for male rats observed at 3.2 h and 1660 to 2120 ng Eq/ml (mean 1960 ng Eq/ml) for female rats observed at 1.4 h. Mean $\text{AUC}_{0-t}$ and $\text{AUC}_{0-\infty}$ values were 65,700 and 67,100 ng Eq $\cdot$ h/ml for male rats and 36,700 and 37,200 ng Eq $\cdot$ h/ml, respectively, for female rats (Table 2).

*Fig. 4. HPLC radiochromatograms of circulating metabolites of lixivaptan (Lixi) in humans after administration of a single oral dose of [14C]lixivaptan.*
C\textsubscript{max} of unchanged lixivaptan ranged from 40 to 180 ng/ml (mean 91 ng/ml) for male rats observed at 0.8 h and 260 to 470 ng/ml (mean 338 ng/ml) for female rats observed at 0.8 h. Mean AUC\textsubscript{0–24} and AUC\textsubscript{0–24} values were 140 and 219 ng · h/ml for male rats and 1530 and 1890 ng · h/ml, respectively, for female rats. The t\textsubscript{1/2} for lixivaptan was 1.3 and 4.2 h for male and female rats, respectively (Table 2). On the basis of AUC values, the majority of the radioactive material in the systemic circulation of rats represents metabolites of lixivaptan.

**Dogs.** Mean plasma concentration–time curves for unchanged lixivaptan and total radioactivity for dogs after a single 10 mg/kg oral dose of [14C]lixivaptan are shown in Fig. 3B. Mean plasma concentration–time curves for unchanged lixivaptan were generated metabolites M7 and M8 each accounted for less than 5% of the relative amount of lixivaptan decreased to 12%, M3 declined slightly, and M6 became the major metabolite, increasing to 42% of the plasma radioactivity. By 24 h postdose, M6 was the only remaining major metabolite, representing 97% of the plasma radioactivity. In contrast, M6 dominated in the male rat plasma at all time points investigated and was the only major metabolite observed (58–74% of the plasma radioactivity). Small amounts of lixivaptan were detected only in the 1-h samples (4% of the plasma radioactivity). In both sexes, M1, M2, M3, M7, and M8 were the minor metabolites and were present only at the earlier time points.

**Circulating Metabolites of Lixivaptan. Humans.** Metabolite profiles of lixivaptan were generated from the plasma of human subjects receiving a single oral 100-mg dose of [13C]lixivaptan at 0.5, 2, 4, 8, 12, 24, and 36 h after dosing (Fig. 4). The relative abundance of metabolites is presented in Table 3. A total of seven radioactive peaks corresponding to nine metabolites were detected in the chromatograms. The pyrrolinone metabolite (M3) could not be resolved because it coeluted with two other metabolites (M1 and M2). At 0.5 h, unchanged drug was the major component in the profile, comprising 43.5% of the plasma radioactivity. Small amounts (mean values of 1–4% of the radioactivity) of the pyrolocarboxylic (M5) and anthranilic acid (M6) metabolites were observed. By 2 h, M5 and M6 accounted for ~17 and ~13% of the plasma radioactivity, respectively, and lixivaptan accounted for 13% of the radioactivity. The relative amount of M5 remained the same at 4 h, after which it declined gradually to 8% at 36 h. M6 increased in relative contribution with time, peaked at 24 h with 39% of the radioactivity, and remained nearly constant (35%) at 36 h. Parent drug was detected at all times assayed. Numerous polar metabolites were present in the 0.5 through 12 h samples. The pyrrolidine isomer (M4) as well as the monooxygenated metabolites M7 and M8 each accounted for less than 5% of the radioactivity at all times examined (Table 3).

**Relative abundance of circulating metabolites in rats and dogs**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Male Rats</th>
<th>Female Rats</th>
<th>Dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>8 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Parent</td>
<td>4.1 ± 0.4</td>
<td>29.9 ± 4.2</td>
<td>12.2 ± 2.2</td>
</tr>
<tr>
<td>Unknown*</td>
<td>1.6 ± 0.2</td>
<td>2.0 ± 0.2</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td>M1</td>
<td>7.0 ± 1.3</td>
<td>4.2 ± 1.6</td>
<td>2.9 ± 5.0</td>
</tr>
<tr>
<td>M2</td>
<td>3.0 ± 0.5</td>
<td>4.5 ± 1.1</td>
<td>2.1 ± 1.8</td>
</tr>
<tr>
<td>M3</td>
<td>5.4 ± 1.5</td>
<td>36.2 ± 2.4</td>
<td>25.2 ± 8.3</td>
</tr>
<tr>
<td>M6</td>
<td>58.5 ± 0.4</td>
<td>90.0 ± 0.8</td>
<td>42.4 ± 12.3</td>
</tr>
<tr>
<td>M7, M8</td>
<td>2.8 ± 0.6</td>
<td>4.5 ± 0.5</td>
<td>2.8 ± 2.5</td>
</tr>
</tbody>
</table>
All three human metabolites (M3, M5, and M6) have adequate cov-
determined in a 9-month dog study and a 2-week rat toxicology study. To further mitigate a metabolite-related risk, the detected in both rats and dogs but was not quantified because of a low level of radioactivity. The combined exposure of metabolites M7 and M8 was 652 ng
respectively, and 13,070 and 22,400 ng Eq
metabolites in humans were found in at least one of the toxicology species studied to facilitate the interpretation of the mass spectra of its metabolites. In the LC-MS spectrum of lixivaptan, a protonated molecular ion, \([M + H]^+\), was observed at \(m/z\) 244. The MS/MS spectrum of the \([M + H]^+\) showed fragment ions at \(m/z\) 395 [MH − methylenepyrrrole] \(^{-}\), 290, 185, 183, 155, 137, 132, and 80 (Fig. 7; Table 1). One of the major fragments at \(m/z\) 290 resulted from loss of the pyrrolobenzodiazepine by cleavage of one amide bond. Cleavage of both amide bonds produced a major fragment at \(m/z\) 137 (carbon-
fluoromethylbenzene) and the less abundant ion at \(m/z\) 155 (carbon-
ylfluoromethylbenzene) and the less abundant ion at \(m/z\) 137 (carbon-
fluoromethylbenzene) and the less abundant ion at \(m/z\) 155 (carbon-
ylfluoromethylbenzene). Other product ions of low abundance were due
to methyleneypyrrrole [\(m/z\) 80], fluoromethylbenzene [\(m/z\) 109], benzyl-N=C=O [\(m/z\) 132], pyrrolobenzodiazepine [\(m/z\) 185 and 183], and [MH − methyleneypyrrrole] \(^{-}\) [\(m/z\) 395].

Assessment and Comparison of Exposure of Human Circulating Metabolites in Toxicology Species. In the rat, dog, and human \(^{14}\)C ADME studies, a number of metabolite peaks, some of which contained more than one metabolite, were observed in plasma from HPLC radiochromatograms. On the basis of chromatographic retention time, all of the metabolites were more polar than lixivaptan. A total of eight metabolites were identified in human plasma; all of them exceeded 10% of the parent exposure (2.0–41% of total radioactivity) as measured by the ratio of the percentage of radioactivity of the metabolite to the percentage of the radioactivity of the parent drug (Tables 3 and 4). In humans, three radioactive peaks were determined to be the most abundant (>25% of plasma radioactivity), whereas other metabolite peaks were quantitatively less abundant. All of the major circulating metabolites (>25% of plasma radioactivity) identified in humans were found in at least one of the toxicology species and in most cases in more than one species. To assess the coverage of metabolites, the exposures of the metabolites in humans and preclinical species were estimated using the equation as follows (Dalvie et al., 2010):

\[
\frac{AUC_{(0-t)}}{AUC_{0}} = \frac{\% \text{ of metabolite}}{\% \text{ of total radioactivity}}
\]

The estimated exposures of lixivaptan and metabolites in humans and preclinical species are depicted in Table 5 and Fig. 5. The exposures (\(AUC_{\text{eq}}\)) of two abundant peaks, M1 + M2 + M3 and M6, were 8100 and 5180 ng Eq · h/ml, respectively, in humans and the estimated exposures in female rats were 7960 and 11,100 ng Eq · h/ml, respectively, and 13,070 and 22,400 ng Eq · h/ml, respectively, in dogs. The combined exposure of metabolites M7 and M8 was 652 ng Eq · h/ml in humans, 5640 ng Eq · h/ml in dogs, 432 ng Eq · h/ml in male rats, and 1050 ng Eq · h/ml in female rats (Table 5). M5 was detected in both rats and dogs but was not quantified because of a low level of radioactivity. To further mitigate a metabolite-related risk, the exposures of three most abundant metabolites (M3, M5, and M6) were determined in a 9-month dog study and a 2-week rat toxicology study. All three human metabolites (M3, M5, and M6) have adequate cov-
erage at the no observable adverse effect level dose in both rats and
dogs (data not shown).

Estimation of the Exposure of Human Metabolites at Steady State. Plasma samples from humans were profiled at 0.5, 2, 4, 8, 12, 24, and 36 h after dosing. The plasma concentrations of lixivaptan and metabolites, as nanogram equivalents per milliliter, for each time point were calculated by multiplying the percentage of individual metabolite by the concentration of total radioactivity. The mean concentration-time curves for lixivaptan and its major metabolites are presented in Fig. 6. These data suggested that M3 (combined with M1 and M2), M5, and M6 were the major circulating metabolites, with exposure ranges from 1.4 to 4.3 times the parent exposure. The \(C_{\text{max}}\) and AUC values from the single dose were modeled to assess the exposure of circulating metabolites at steady state, and calculated values are shown in Table 6. The steady-state AUC values for lixivaptan, M5, and M6 were estimated to be 2440, 3230, and 8110 ng Eq/ml, respectively (Table 6). The combined exposure of M1, M2, and M3 was 10,700 ng Eq · h/ml. The exposures of these three metabolites in humans at the steady state were also determined by a validated LC-MS/MS method in plasma from a 7-day clinical study. The observed exposures of the parent drug, M5, and M6 were in good agreement with the predicted values. The exposure of M3 suggested that it was a minor drug-derived product in plasma at the steady state. The amount of M3 after a single dose in the \(^{14}\)C study was overestimated because the chromatographic peak of M3 in the radiochromatograms coeluted with several peaks including metabolites (M1 and M2).
The presence of the above product ions in a mass spectrum of a metabolite, with the exception of $m/z$ 183, indicated that their corresponding substructures were unchanged in a metabolite molecular structure. Therefore, sites of metabolism on the lixivaptan molecule could be localized to the benzyl and pyrrole–CH$_2$– parts of the pyrrolobenzodiazepine moiety. Therefore, this metabolite was formed by two oxidations of the pyrrolobenzodiazepine moiety. Therefore, M2 was formed by oxidation and addition of a water molecule to the pyrrolobenzodiazepine moiety. It is speculated that the second oxidation site as well as the site of the additional two hydrogen atoms was also on the pyrrolobenzodiazepine moiety. The MS/MS spectrum of [M + H]$^+$ showed fragment ions at $m/z$ 290, 155, and 109, indicating the oxidation at the pyrrolobenzodiazepine moiety (Table 7). M3 was identified as N-[3-chloro-4-(3-oxo-2,3-dihydro-1H-SH-benzo[3]pyrrolo[1,2-a][1,4]diazepine-10-carbonyl-phenyl]-5-fluoro-2-methyl-benzamide (WAY-138451) because it had the same HPLC retention time and product ion spectrum as synthetic WAY-138451.

Metabolite M4. The LC-MS mass spectrum of M4 displayed a [M + H]$^+$ at $m/z$ 490, 16 Da heavier than lixivaptan, suggesting the addition of one oxygen atom to lixivaptan. The LC-MS/MS spectrum of [M + H]$^+$ included product ions at $m/z$ 290, 183, 155, 132, and 109, indicating the oxidation at the pyrrolobenzodiazepine moiety (Table 7). The product ion at $m/z$ 199 indicated the addition of an oxygen atom to the pyrrolobenzodiazepine moiety. The retention time, UV spectra, and mass spectra of M6 were similar to those of an authentic standard, synthetic 1-(2-[2-chloro-4-(5-fluoro-2-methyl-benzoyl)-5-fluoro-2-methyl-benzamide (WAY-137930).

Metabolite M5. The LC-MS mass spectrum for M5 displayed a [M + H]$^+$ at $m/z$ 506, 32 Da heavier than lixivaptan, suggesting the addition of two oxygen atoms to lixivaptan. The LC-MS/MS spectrum of [M + H]$^+$ showed fragment ions at $m/z$ 155, 137, 199, and 290, indicating metabolism in the pyrrolobenzodiazepine moiety (Table 7). The product ion at $m/z$ 199 indicated the addition of an oxygen atom to the pyrrolobenzodiazepine moiety. The second oxidation site as well as the site of the additional two hydrogen atoms was also on the pyrrolobenzodiazepine moiety. Therefore, M2 was formed by oxidation and addition of a water molecule to the pyrrolobenzodiazepine moiety. It is speculated that M2 is formed by oxidation of one of the hydroxylated metabolites (M3, M4, or M8) at the diazepine moiety to form an unstable carbonylamine, which could then decompose to an aldehyde. Reduction of the aldehyde to alcohol would form M2.

**Table 6**

Calculated exposure of lixivaptan and metabolites in humans

<table>
<thead>
<tr>
<th>Analyte</th>
<th>$^{14}$C Human Study</th>
<th>$^{14}$C Simulated Study</th>
<th>Nonradioanalytical Clinical Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C_{max}$</td>
<td>AUC</td>
<td>$C_{ss}$</td>
</tr>
<tr>
<td>Lixivaptan</td>
<td>454 ± 114</td>
<td>1860 ± 305</td>
<td>575 ± 170</td>
</tr>
<tr>
<td>M3$^a$</td>
<td>716 ± 317</td>
<td>8100 ± 2470</td>
<td>942 ± 349</td>
</tr>
<tr>
<td>M5</td>
<td>290 ± 99</td>
<td>2680 ± 591</td>
<td>346 ± 108</td>
</tr>
<tr>
<td>M6</td>
<td>278 ± 103</td>
<td>5180 ± 2410</td>
<td>428 ± 160</td>
</tr>
</tbody>
</table>

$^a$ Steady state:

$^a$ Quantified with M1 and M2 in the $^{14}$C human AME study.
amino)benzoylamino-benzyl)-1H-pyrrole-2-carboxylic acid (WAY-141624) had the same molecular weight, HPLC retention time, and MS/MS spectrum as the M5 metabolite. Therefore, M5 was identified as WAY-141624, a pyrrolocarboxylic acid of lixivaptan.

**Metabolite M6.** The LC-MS mass spectrum of M6 displayed a [M + H]^+ at m/z 427, a loss of 47 Da from lixivaptan. The MS/MS spectrum of [M + H]^+ included product ions at m/z 290, 155, 137, and 109, which all indicated that the nonpyrrolobenzodiazepine portions of lixivaptan were unchanged (Table 7). The HPLC retention time and UV spectrum of M6 matched with those of a synthetic standard 2-[2-chloro-4-(5-fluoro-2-methyl-benzoylamino)-benzoylamino]-benzoic acid (WAY-138758). Therefore, M6 was identified as WAY-138758, the anthranilic acid metabolite of lixivaptan.

**Metabolite M7.** The LC/MS mass spectrum of M7 displayed a [M + H]^+ at m/z 490, 16 Da higher than lixivaptan, suggesting the addition of one oxygen atom to the parent molecule. The LC-MS/MS spectrum of [M + H]^+ showed fragment ions at m/z 155 and 137, suggesting that the carbonylfluoromethylbenzene and the pyrrole-CH2- were unchanged (Table 7). The product ion at m/z 80 indicated that the pyrrole-CH2- portion of the pyrrolobenzodiazepine moiety was not metabolized. The product ion at m/z 393 represented a net loss of 2 Da from the corresponding product ion at m/z 395 of lixivaptan. This mass difference was due to oxidation of lixivaptan to generate the metabolite, which then lost H2O from [MH – methylenepryrole]^+ (m/z 411, not observed) via fragmentation in the products of the [M + H]^+ experiment. This indicated the addition of a hydroxyl group on the benzyl carbon of the pyrrolobenzodiazepine moiety. Therefore, M7 was probably formed by hydroxylation at the 5-position of the pyrrolobenzodiazepine moiety of lixivaptan.

**Metabolite M8.** The LC-MS mass spectrum of M8 showed the [M + H]^+ at m/z 490, which was 16 Da heavier than lixivaptan and suggested a single oxidation of lixivaptan. The MS/MS spectrum of [M + H]^+ gave fragment ions at m/z 137 and 80, suggesting that the carbonylfluoromethylbenzene and the pyrrole-CH2- were unchanged (Table 7). Therefore, M8 is formed by oxidation of either the chlorophenyl or benzyl moieties of lixivaptan.

### Discussion

The comparison of metabolite profiles of an investigational drug in preclinical species and humans is essential to ensure that animal species used for long-term safety assessment are appropriate models for humans and that all major human metabolites (≥10% of total plasma radioactivity) have adequate coverage in these species [U.S. Food and Drug Administration, Guidance for Industry: Safety Testing of Drug Metabolites, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm079266.pdf; International Conference on Harmonization, Non-Clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals M3 (R2), http://www.emea.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002720.pdf; Robison and Jacobs, 2009]. The other reason for conducting radiolabeled ADME studies in toxicology species is also to understand species-dependent metabolism. For example, efavirenz, a potent non-nucleoside reverse transcriptase inhibitor widely prescribed for the treatment of human immunodeficiency virus infection, produces renal tubular epithelial cell necrosis in rats but not in monkeys or humans. The radiolabeled ADME studies provided compelling evidence that a species-specific formation of glutathione conjugate(s) from efavirenz was responsible for the nephrotoxicity in rats (Mutlib et al., 2000). Definitive ADME studies using a radiolabeled drug provide a comprehensive and quantitative profile of both excretory and circulating metabolites (Penner et al., 2012).

In the present study, the metabolism of lixivaptan in toxicology species and humans was characterized from mass balance and excretion studies using [14C]lixivaptan. The exposures of human metabo-
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>MH&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Structure</th>
<th>Major Fragment Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lixivaptan</td>
<td>490</td>
<td></td>
<td>395, 290, 185, 183, 155, 137, 109, 80</td>
</tr>
<tr>
<td>M1</td>
<td>506</td>
<td></td>
<td>290, 155, 137, 109, 96</td>
</tr>
<tr>
<td>M2</td>
<td>508</td>
<td></td>
<td>290, 155, 137</td>
</tr>
<tr>
<td>M3</td>
<td>490</td>
<td></td>
<td>290, 155, 137, 109</td>
</tr>
<tr>
<td>M4</td>
<td>490</td>
<td></td>
<td>290, 199, 155, 137</td>
</tr>
<tr>
<td>M5</td>
<td>506</td>
<td></td>
<td>290, 155, 137, 109</td>
</tr>
</tbody>
</table>
Metabolites were compared with those of the rats and dogs obtained from the single-dose radiolabeled ADME studies. In addition, the steady-state exposures (C_{max} and AUC) of three abundant metabolites (M3, M5, and M6) were calculated in humans from the single-dose study and compared with the exposures obtained from a 7-day clinical study using validated LC-MS/MS assay.

After oral administration to rats, dogs, and humans, the majority of the radioactivity was eliminated in the feces, suggesting that fecal/biliary excretion was the primary route of elimination of [14C]lixivaptan-derived radioactivity in these species. A separate study in the bile duct-cannulated rats further suggested that biliary excretion was the major route of elimination of lixivaptan and metabolites in rats. Profiling of fecal samples from humans showed that unchanged drug represents only 13.5% of the dose, suggesting that lixivaptan is also well absorbed in humans and the radioactivity in feces was due to its biliary excretion rather than to unabsorbed drug. The absorption of lixivaptan was rapid in both preclinical species and humans, because the plasma concentrations for lixivaptan and total radioactivity peaked within 3 h after oral administration. The plasma concentrations of lixivaptan were much lower than those of total radioactivity, indicating that the majority of the radioactive material in the plasma represents metabolites in all species.

Metabolite identification was performed by mass spectrometry combined with chromatographic retention time. Comparison of MS/MS fragmentation patterns and retention times of authentic standards was used for definitive identification when possible. The metabolic pathways of lixivaptan are depicted in Fig. 1. The major metabolites were the pyrrolocarboxylic (M5) and anthranilic (M6) acids of lixivaptan, with the latter having a more prolonged systemic presence. Whereas M6 was a major circulating metabolite in all species studied, M5 was very minor in the rat and dog. The pyrroline-none metabolite (M3) was observed as a major metabolite in rats and dogs, but its significance is unclear in humans because of interference with another incompletely resolved metabolites. Most of the metabolites characterized were formed by single or multiple oxidations to the pyrrolobenzodiazepine headpiece of the molecule. In vitro studies using human liver microsomes indicated that CYP3A4 was the primary enzyme responsible for the formation of all of the known metabolites of lixivaptan. None of the metabolites that have been tested (M3, M4, M5, and M6) have shown any significant pharmacological activity (Molinari et al., 2007).

For quantitative profiling of metabolites in human circulation, plasma samples were profiled separately for each subject at 0.5, 2, 4, 8, 12, 24, and 36 h after dosing, which allows for calculation of the PK parameters of the parent drug and metabolites and helps in simulating their exposures (C_{max} and AUC) at steady state to eliminate the need for analyzing samples after multiple doses of the drug. A number of metabolite chromatographic peaks, some of which contained more than one metabolite, were observed in plasma from HPLC radiochromatograms. Based on chromatographic retention times, all of the metabolites were more polar than lixivaptan.

The abundance and the exposure (AUC) of three metabolites in humans appear to be >10% of total radioactivity exposure. The two most abundant metabolites (M5 and M6) appear to be >100% of the parent AUC. Quantitation of M3 was not possible because it was incompletely resolved from other metabolites (M1 and M2). Six other metabolites appear to be <10% of the total drug-related material (∼15–20% of the parent AUC). On the basis of the single-dose

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>MH°</th>
<th>Structure</th>
<th>Major Fragment Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6</td>
<td>427</td>
<td><img src="image" alt="" /></td>
<td>290, 155, 137, 109</td>
</tr>
<tr>
<td>M7</td>
<td>490</td>
<td><img src="image" alt="" /></td>
<td>290, 137, 80</td>
</tr>
<tr>
<td>M8</td>
<td>490</td>
<td><img src="image" alt="" /></td>
<td>183, 137, 80</td>
</tr>
</tbody>
</table>
radiolabeled ADME studies, the exposure of three human metabolites (M6, M7, and M8) was highest at least in one preclinical species (Table 5; Fig. 4). In addition, the combined exposure of metabolites (M1, M2, and M3) was greater in both rats and dogs than in humans (Table 5; Fig. 4). Exposure of two less abundant metabolites in preclinical species could not be determined. The exposures of three metabolites (M3, M5, and M6) estimated from nonradiolabeled studies in humans and preclinical toxicology species using the LC-MS/MS method also confirmed that the human metabolites that exceeded >10% of total radioactivity have adequate coverage in toxicology species. An unidentified peak and M4 were present <5% of total circulating radioactivity (Table 5) and, therefore, are not a safety concern.

The exposure of lixivaptan and three most abundant metabolites (M3, M5, and M6) was modeled to estimate their exposure at steady state. The observed exposures of the parent drug, M5, and M6 were in good agreement with the predicted values. The LC-MS/MS analysis of metabolite M3 suggested that M3 is just a minor drug-derived product in plasma at steady state. The amount of M3 after a single dose in the 14C ADME study was overestimated because of its coelution with other drug-derived material. Therefore, it is very important to develop an adequate separation (HPLC or ultra-HPLC) method that can resolve all metabolites. Recent developments in separation technology and specifically in ultra-HPLC columns together with compatible pumps and detectors have drastically increased resolution and sensitivity of analysis. The use of this method to estimate steady-state PK parameters is based on the assumption that the pharmacokinetics of lixivaptan or its metabolites are not time-dependent.

In summary, lixivaptan is extensively metabolized in humans to numerous metabolites, most of which have undergone one or more oxidations to the headpiece of the molecule. The human circulating metabolites that exceed 10% of total drug-related material in plasma had equal or greater exposure at least in one animal species used for long-term safety assessment at the highest safe dose. The exposure data from this study showed that all of the human circulating metabolites exceeding 10% of total drug-related material in plasma have greater or equal exposure in at least one of the toxicology species and in most cases in more than one species. Therefore, a well designed radiolabeled human study can help in predicting metabolite-related liability and in addressing all major MIST-related issues. The basic requirements to simulate the exposure from a single dose study are 1) adequate time points for blood collection to calculate the t1/2 of each metabolite, 2) the baseline HPLC separation of each metabolite for the quantitation by radioactivity measurements, and 3) no induction and/or time-dependent inhibition of metabolism.

Acknowledgments

We thank Drs. Liyu Yang and Ron Huang for analysis of metabolites from nonradiolabeled studies and Drs. Lin Xu, Natasha Penner, and Tonika Bohnert for help with calculating PK parameters and modeling steady-state exposures of metabolites.

Authorship Contributions

Participated in research design: Prakash, Li, Orlandi, and Klunk.
Conducted experiments: Li.
Contributed new reagents or analytic tools: Prakash and Li.
Performed data analysis: Prakash and Li.
Wrote or contributed to the writing of the manuscript: Prakash, Li, Orlandi, and Klunk.

References


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