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Assessment of Metabolites Exposure in Preclinical Species and Humans at Steady State from the Single Dose Radiolabelled ADME Studies: A Case Study

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Running Title: Assessment of metabolites exposure at steady state

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Abbreviations: NME, new chemical entity; CYP, cytochrome P450; MIST, metabolite in safety teting ; TRA total radioactivity; radio-HPLC, HPLC with on-line radioactivity detector; LC-MS/MS, liquid chromatography-tandem mass spectrometry; CID, collision induced dissociation; LSC, liquid scintillation counting

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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 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. Following an oral dose of [^{14}C]lixivaptan to humans, a total of nine metabolites were detected in systemic circulation; eight of them exceed 10% of the parent exposure (2 to 41% of total radioactivity). The plasma samples were profiled for all subjects at each time point by HPLC and metabolites were quantified using a radioactive detector. Based on single dose AUC values, exposure of six human metabolites was greater at least in one preclinical species used in toxicology evaluation. Based on $T_{1/2}$ of lixivaptan and two major metabolites from single dose in humans, their AUC and C_{max} values were simulated at the steady state. The simulated exposures (C_{max} and AUC) values of parent drug and the two most abundant metabolites were similar to those of from a 7 day clinical study obtained using a validated LC-MS/MS assay, suggesting that a well-designed single dose radiolabeled human study can help in addressing the metabolites in safety testing-related issues.

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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 (Food and Drug Administration). 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 (FDA, 2008). If this is not achieved, then additional toxicological testing on metabolites may be required to achieve the recommended exposure to the metabolite(s). This FDA guidance sparked a debate among the regulators and the industry and was critically evaluated in several publications (Davis-Bruno and Atrakchi, 2006; Smith and Obach, 2006, 2009; Prueksaritanont et al., 2006; Smith et al., 2009; Leclercq et al., 2009; Robinson and Jacobs, 2009). The more recent International Conference on Harmonization guidance 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 (ICH, 2009).

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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 authentic standards (Vishwanathan et al., 2009; Ma et al., 2010; Gao et. al., 2010). These approaches are useful to compare the metabolic profiles between humans and preclinical species but may not provide their exposure since establishing a correlation between an MS signal for a metabolite to its actual amount is difficult without a synthetic standard (Ramanathan et al., 2007; Penner et al., 2009). In addition, for an extensively metabolized drug, there may be several metabolites greater than 10% of total AUC and therefore, require considerable efforts and investment on 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 the definitive data on the metabolic profiles and relative abundance of metabolites in plasma and urine (Penner et al., 2009). 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]-benzodiazopin-10(11H)-ylcarbonyl)-3-chlorophenyl]-benzamide) is a potent, selective, orally active, non-peptide competitive vasopressin V2 receptor antagonist (Chan et al.,1998; Ashwell et al., 2000; Albright et al., 1998; Zmily et al., 2011). It is currently being developed for the treatment of hyponatremia associated with SIADH (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 neurohormonal system,

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suggesting that it has a therapeutic potential in managing water retention in patients with liver cirrhosis with ascites (Swan et al., 1999; 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 C_{max} 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 compared to 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 circulatory metabolites in toxicology species, rats and dogs. [^{14}C]lixivaptan was orally administered to young healthy male volunteers, Sprague-Dawley rats and dogs, and the metabolic profiles were obtained in plasma. The exposures (C_{max} and AUC) of lixivaptan and its most abundant metabolites from single dose in humans were simulated at the steady state and compared with those obtained from a 7 day clinical study.

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MATERIALS AND METHODS

General Chemicals

Commercially obtained chemicals and solvents were of HPLC or analytical grade. Zorbax Rx-C18 (5 micron; 3.0 x 250 mm) analytical column was purchased from Mac Mod Analytical Inc. (Chadds Ford, PA). Ultima Gold XR, Carbosorb and Permafluor E+ scintillation cocktails were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). HPLC grade acetonitrile, formic acid and water, and certified ACS grade ammonium acetate and acetic acid were obtained from J. T. Baker (Phillipsburg, NJ). Dimethylsulfoxide, dimethylformamide and polyethylene glycol (PEG 200) were obtained from Sigma Chemical Company (St. Louis, MO). Pluronic F68 was obtained from BASF Inc. (Mt. Olive, NJ).

Radiolabeled Drug and Reference Compounds

[^{14}C]Lixivaptan: 5-fluoro-2-methyl-N-[4-(5H-pyrrolo[2,1-C][1,4]-benzodiazopin-10(11H)-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-5,10,11,11a-dihydro-3H-benzo[e] pyrrolo[1,2-a][1,4]diazepine-10-carbonyl)-phenyl]-5-fluoro-2-methyl-benzamide, M4 (WAY-137930: N-[3-chloro-4-(3-oxo-2,3 dihydro 1H,5H benzo[3]pyrrolo [1,2a][1,4]diazepine-10-carbonyl)-phenyl]-5-fluoro-2-methyl-benzamide), M5 (WAY-141624: 1-(2-[2-chloro-4-(5-fluoro-2-methyl-benzoylamino) benzoylamino]-benzyl)-1H-pyrrole-2-carboxylic acid) and M6

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(WAY-138758: 2-[2-chloro-4-(5-fluoro-2-methyl-benzoylamino)-benzoylamino]-benzoic acid were synthesized as described earlier (Molinari et al., 2007; Ashwell et al., 2000).

Study Design, Dosing and sample Collection in Humans. Six healthy male subjects between the ages of 18 to 45 years participated in the study. All subjects provided written, informed consent prior to 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 ICH Good Clinical Practices (GCP) guidelines, the ethical principles that have their origin in the Declaration of Helsinki, and in compliance with the US 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 [^{14}C]lixivaptan (2x 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 their bladder and provided a 50-ml sample of urine immediately prior to dosing, which was frozen at or below -20 ^{o}C. After dosing, urine samples were collected at 0-4, 4-8, 8-12, 12-24h and at 24 h intervals for up to 168 h. All of the urine collected during each time period was mixed, the total volume 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 prior to dosing (0) and

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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 ^{o}C until analyzed.

Animals, Dosing and sample Collection

Intact and bile-duct cannulated (BDC) Sprague Dawley (SD) 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 prior to 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 appropriate for sample collection: Rats designated for collection of bile were housed in individual, suspended, stainless steel cages until the bile cannulae 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 prior to administration of the dose and were fed 4 h after the dose. The animals were provided water *ad libitium*. The drinking water for BDC rats was supplemented with bile salts 1% w/v, NaCl 0.9% w/v, and KCl 0.05% w/v to replenish lost electrolytes during the 48 hour collection of bile. All the animal studies were conducted in a research facility accredited by the American Association for the Accreditation of Laboratory Animal Care.

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

Bile samples were collected from the bile duct cannulated rats (Group 1) at intervals of 0-4, 4-8, 8-24, and 24-48 h. The bile was collected on wet ice. Urine and feces were collected from intact rats (Group 2) at 0-8, 8-24 and at every 24 h intervals 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_2EDTA anticoagulant via 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. Following removal of each blood sample starting with 1 h sample, animals received 1 ml of donor blood to maintain blood volume and hematocrit. Blood samples (~10 ml) from 3 rats/time point/gender (Group 4) were collected by cardiac puncture under isoflurane anesthesia in 10 ml K_2EDTA 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 ^{o}C until analysis.

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Dogs. One group of four male dogs received an oral (10 mg/kg, 2 ml/kg) dose of [^{14}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}C]lixivaptan and pluronic F68 in deionized water. The specific activity of dosing formulation was 1.04 μCi/mg.

For pharmacokinetics of total radioactivity and lixivaptan, blood samples (5 ml) were collected into tubes containing K_2EDTA anticoagulant via jugular vein from dogs at 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48, 72, 120 1nd 168 h postdose. An additional blood (10 ml) was into tubes containing K_2EDTA anticoagulant 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 0-8, 8-24 and at every 24 h intervals for 7 days. The first fecal sample was collected at 24 h postdose. All of the biological samples were stored at -20 ^{o}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. Following homogenization, duplicate aliquots were transferred into tarred cones and pads, weighed, dried overnight at ambient temperature before combustion in a Model 307-D Tri-Carb Sample Oxidizer (Canberra-Packard Co.) equipped with an Oximate-80 Robotic Automatic Sampler (PerkinElmer Life Sciences, Inc.; Boston, MA). Radioactivity in the combustion

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products was determined by trapping the liberated CO_2 in Carbo-sorb (PerkinElmer Life Sciences, Inc.; Boston, MA) followed by liquid scintillation counting using Permafluor E+ (PerkinElmer Life Sciences, Inc.; Boston, MA) as a scintillation cocktail. Combustion efficiency was determined daily, prior to the combustion of study samples, using diluted dose formulation or a carbon-14 standard. The measured radioactivity content in combusted samples was adjusted using the combustion efficiency values.

Samples collected prior to 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 ng-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 about 10 min and the supernatants placed in clean 50 ml Falcon polypropylene conical tubes. The pellets were extracted with additional 2 volumes of acetonitrile, the mixtures were centrifuged at 3000 rpm for about 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 Turbo Vap LV evaporator (Caliper life sciences,

Hopkinton, MA). The residues were then reconstituted in acetonitrile, and the equal amount of water was added. Aliquots (100 µl) were injected onto the HPLC system.

High Performance Liquid Chromatography (HPLC)

The HPLC system consisted of a model 600-MS pump (Waters Instruments, Milford, MA) connected to a spectro-Monitor 3200 variable wavelength detector: (monitoring 270 nm; LDC/Milton Roy, Riviera Beach, FL), a model 715 Ultra WISP automatic sampler (Waters Instruments), a radiomatic radioactivity flow detector model Flo-One Beta A525 (Packard Instruments, Meriden, CT) and a HP3393A integrator (Hewlett-Packard, San Fernando, CA). For rat and dog plasma, chromatography was performed on a Zorbax Rx-C18 (5 micron; 3.0 x 250 mm) analytical column (Mac Mod Analytical Inc.,Chadds Ford, PA) with a mobile phase containing a mixture of acetonitrile:10 mM ammonium formate (29:71; solvent A), 10 mM ammonium formate (solvent B) and acetonitrile: 10 mM ammonium formate (90:10; solvent C). The mobile phase was initially composed of solvent A/solvent B/solvent C (70:30:0). The mobile phase composition was then linearly programmed to solvent A/solvent B/solvent C (100:0:0), over 30 min and then changed to solvent A/solvent B/solvent C (8:0:92) over 30 min. The mobile phase composition was kept for 3 min and then returned to the starting solvent mixture over 1 min. The system was allowed to equilibrate for approximately 10 min before making the next injection. The flow rate was 0.5 ml/min and the separation was achieved at ambient temperature.

For human plasma, chromatography was performed on a Zorbax Rx-C18 (5 micron; 3.0 x 250 mm) analytical column (Mac Mod Analytical Inc., Chadds Ford, PA) with a mobile phase consisting of 0.2% formic acid in acetonitrile:water (solvent A, 29:71 and solvent B, 90:10).

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The mobile phase was initially composed of solvent A/solvent B (100:0). The mobile phase composition was then linearly programmed to solvent A/solvent B (75:25), over 15 min. It was then returned to the starting solvent mixture over 1 min. The system was allowed to equilibrate for approximately 10 min before making the next injection. The flow rate was 1 ml/min and the separation was achieved at ambient temperature.

Quantitation of Metabolites

Metabolite quantification was performed using a radioactivity flow detector model Flo-One Beta A525 using radiomatic v3.01 software (Packard Instruments). The radiometric detector provided integrated peak representation in CPM as well as the percentage of total radioactivity comprised by the each peak within the radiochromatogram. The detector was operated in the homogeneous liquid scintillation counting mode with the addition of 2 ml/min Ultima Flo M scintillation fluid.

LC-MS/MS

Identification of the metabolites was performed on a Sciex API III+ triple quadrupole mass spectrometer (AB-Sciex, Foster City, CA) operating with an electrospray ionization (ESI) interface. The interface was operated at 5000 V and the spectrometer was operated in the positive ion mode with capillary temperature set at 250ºC; nebulizer gas 75-85 psi; curtain gas flow 1.0-1.4 l/min; interface temp. 50-60 °C; declustering potential 55-70 V; collision gas thickness 150-250 x1012 atoms/cm^2, collision offset -35 eV, resolution (Q1 and Q3) 0.7 Da ± 0.2 Da at 50% peak height.

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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 (d4-lixivaptan, d4-M5 and d4-M6) analogs were used as the internal standards. Multiple reaction monitoring (MRM) transitions for lixivaptan and M5 and M6 were 474.1→290.0, 490.1.1→290.0 and 427.1→290.0, respectively. The corresponding MRM 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 5-2000 ng/ml in human plasma**.**

Pharmacokinetic Analysis

Pharmacokinetic parameters were determined using the WinNonlin Professional Software (version 5.3; Pharsight Corp., Mountain View, CA). Maximum observed concentrations (C_{max}) of lixivaptan or total radioactivity (parent drug equivalents) in plasma were estimated directly from the experimental data, with t_{max} defined as the time of first occurrence of C_{max}. Terminal phase rate constants (k_{el}) were estimated using least squares regression analysis of the Serum concentration-time data obtained during the terminal log-linear phase. Half-life ($t_{1/2}$) was calculated as $0.693/k_{el}$. 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 linear trapezoidal rule. AUC from time t to infinity ($AUC_{(t-\infty)}$) was estimated as C_{est}/k_{el} where C_{est} represents the estimated concentration at time t based on the aforementioned regression analysis. AUC from

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time 0 to infinity ($AUC_{(0-\infty)}$) was estimated as the sum of $AUC_{(0-t)}$ and $AUC_{(t-\infty)}$ values. C_{max} and AUC of lixivaptan and its metabolites at steady-state were estimated based on $AUC_{(0-24h)}$ after first dose and the accumulation factor derived from elimination half-life $t_{1/2}$ using the equation of R=1/(1-exp(-tau*(0.693/t1/2))). C_{max} and AUC of lixivaptan and its metabolites at steady-state were estimated by multiplying the values after single dose by its respective R. 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.

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 administration. A mean total of 84.2% of the radioactivity was excreted in the urine and feces over a period of 168 h postdose administration. 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 SD 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 70-80% of the recovered radioactivity being excreted in the first 8 h.

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Dogs. The radioactive dose was quantitatively recovered in dogs after oral dose (96.0%) over a period of168 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 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 following oral administration [^{14}C]lixivaptan are graphically depicted in figure 2. [^{14}C]lixivaptan was rapidly absorbed with a t_{max} of radioactivity in plasma of 1.2 h. The individual values for C_{max} of total radioactivity ranged from1,110 ng.equivq/ml to 2670 ng.equiv/ml (mean of 1,770 ng.equiv/ml). Mean $AUC_{0\text{-}tlast}$ and $AUC_{0\text{-}\infty}$ values were 24,700 ng equiv.h/ml and 26,200 ng equiv.h/ml, respectively (Table 2).

C_{max} values for unchanged lixivaptan ranged from 306 ng/ml to 609 ng/ml with a mean of 437 ng/ml and occurred within 1 h after dose administration. Mean $AUC_{0\text{-}tlast}$ and $AUC_{0\text{-}\infty}$ values were 1,540 ng.h/ml and 1,570 ng.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_{max}), and 0.06 ($AUC_{t0\text{-}tlast}$ and $AUC_{0\text{-}\infty}$). These large differences indicate that the majority of the radioactive material in the systemic circulation represents metabolites of lixivaptan and some of these metabolites are eliminated at slower rates than the parent compound.

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

C_{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 $AUC_{0\text{-tlast}}$ and $AUC_{0\text{-}\infty}$ values were 140 and 219 ng.h/ml for male rats and 1530 and 1890 ng .h/ml, respectively, for female rats. The $T_{1/2}$ for lixivaptan was 1.3 and 4.2 h for male and female rats, respectively (Table 2). Based on 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 following a single 10 mg/kg oral dose of [^{14}C]lixivaptan are shown in figure 3B. C_{max} of radioactivity ranged from 3020 to 4920 ng equiv/ml (mean 3570 ng equiv/ml) observed at 1.8 h. Mean $AUC_{0\text{-tlast}}$ and $AUC_{0\text{-}\infty}$ values were 82,800 and 94,900 ng equiv.h/ml, respectively (Table 2).

C_{max} of unchanged lixivaptan ranged from 410 to 950 ng/ml (mean 759 ng/ml) observed at 0.8 h. Mean $AUC_{0\text{-tlast}}$ and $AUC_{0\text{-}\infty}$ values were 4700 and 4870 ng .h/ml, respectively. The $T_{1/2}$ of

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lixivaptan was 8.8 h (Table 2). Based on AUC values, lixivaptan accounted only <6% of the total circulating radioactivity.

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 [^{14}C]lixivaptan at 0.5, 2, 4, 8, 12, 24 and 36 h after dosing (Figure. 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 since it co-eluted 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 to 4 % of the radioactivity) of the pyrrolocarboxylic (M5) and anthranilic acid (M6) metabolites were observed. By 2 h, the 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, and 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 h through 12 h samples. The pyrrolinone 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).

Rats. Metabolite profiles of lixivaptan from the plasma of rats receiving a single oral 100 mg dose of [^{14}C] lixivaptan were generated at 1, 8, and 24 h after dosing. The relative distribution of metabolites (percent of radioactivity) in male and female rat plasma are shown in Table 4. In

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female rat plasma at 1 h, parent drug and M3 were the prominent peaks and represented ~30 and 36% of the total radioactivity, respectively. M6 represented ~9% of the radioactivity. By 8 h, 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 post dose, 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% to 74 % of the plasma radioactivity). Small amounts of lixivaptan were detected in only the 1 h samples (4% of the plasma radioactivity). In both sexes, M1, M2, M3, M7 and M8 were the minor metabolites and present only at the earlier time points.

Dogs. Metabolite profiles of lixivaptan were generated from the dog plasma following a single 10 mg/kg oral dose of [^{14}C] lixivaptan at 2, 8, and 24 h after dosing. The relative distribution of metabolites in dog plasma is presented in Tables 4. Analysis of dog plasma at 2 h showed that the major components were M2 (14%), M3 (19%), M6 (15% of the radioactivity), M7 and M8 combined (16%) and lixivaptan (21%). At 8 h, M6 doubled in relative percent of radioactivity, and an unknown metabolite emerged as a major component (26% of the radioactivity). M2, M3, M7 and M8 declined by approximately half, and parent drug decreased to 15% of the plasma radioactivity. By 24 h post dose, unknown metabolites and M6 each comprised ~40% of the plasma radioactivity. Lixivaptan was detected in three of the four dogs at 24 h. M5, M7 and M8 were the minor components in the dog plasma.

Assessment and Comparison of Exposure of Human Circulating Metabolites in Toxicology Species

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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. Based on 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 exceed 10% of the parent exposure (2.0 to 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 the human, three radioactive peaks were determined to be the most abundant (>25% of plasma radioactivity) while 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 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).

$$AUC_{(0\text{-tlast})metabolite} = \%\ of\ metabolite \times AUC_{(0\text{-tlast})total\ radioactivity}$$

The estimated exposures of the lixivaptan and metabolites in humans and preclinical species are depicted in Table 5 and Figure 5. The exposures ($AUC_{0–tlast}$) of two abundant peaks, M1+M2+M3, and M6 were 8100, and 5180 ng-equiv-h/ml, respectively, in humans and the estimated exposures in the female rat were 7960 and 11100 ng-equiv-h/ml, respectively and 13070 and 22400 ng.equiv.h/ml, respectively, in dogs. The combined exposure of metabolites M7 and M8 was 652 ng.equiv.h/ml, 5640 ng.equiv.h/ml in dogs 1050 in female rats (Table 5). M5 was detected in both rats and dogs but not quantified due to 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-weeks rat toxicology study. All

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three human metabolites (M3, M5 and M6) have adequate coverage at the NOAEL dose in both rats and dogs (not shown).

Estimation 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 ng-equiv/ml, for each time-point were calculated by multiplying the percent of individual metabolite with the concentration of total radioactivity. The mean concentration–time curves for lixivaptan and its major metabolites are presented in figure 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 of the parent exposure. The C_{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 as 2440, 3230 and 8110 ng equiv/ml, respectively (Table 6). The combined exposure of M1, M2 and M3 was 10700 ng equiv.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 ^{14}C-AME study was overestimated because the chromatographic peak of M3 in the radiochromatograms co-eluted with several peaks including metabolites (M1 and M2).

Identification of Metabolites

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Mass Spectral Fragmentation of Lixivaptan Standard

The fragmentation pattern of lixivaptan was 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* 474. The MS/MS spectrum of the [M+H]+ showed fragment ions at *m/z* 395 (MH-methylenepyrrole)$^+$, 290, 185, 183, 155, 137, 132 and 80 (Figure 7, Table 7). 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 (carbonylfluoromethylbenzene) and the less abundant ion at *m/z* 155 (carbonylchloroaniline). Other product ions of low abundance were due to methylenepyrrole (*m/z* 80), fluoromethylbenzene (*m/z* 109), benzyl-N=C=O (*m/z* 132), pyrrolobenzodiazepine (*m/z* , 185, 183) and [MH-methylenepyrrole]$^+$ (*m/z* 395).

The presence of 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-CH2- parts of the pyrrolobenzodiazepine, the carbonylfluoromethylbenzene and to the chloroaniline moieties.

Metabolite M1. The LC/MS mass spectrum of M1 displayed $[M+H]^+$ at *m/z* 506 which suggested the addition of two oxygen atoms to lixivaptan. The MS/MS spectrum of $[M+H]^+$ showed fragment ions at *m/z* 290, 155, 109 and 96 (Table 7). The ions at *m/z* 290, 155 and 109 suggested that the non-pyrrolobenzodiazepine portion of the lixivaptan molecule was unchanged. The product ion at *m/z* 96, 16 Da higher than the corresponding lixivaptan product ion at *m/z* 80,

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suggested that the monooxygenation had occurred on the pyrrole-CH2- portion of the pyrrolobenzodiazepine. Therefore, this metabolite was formed by two oxidations of the pyrrolobenzodiazepine moiety.

Metabolite M2. M2 displayed a $[M+H]^+$ at *m/z* 508, 34 Da heavier than lixivaptan, suggesting the addition of two oxygen and two hydrogen atoms. The MS/MS spectrum of $[M+H]^+$ contained products ions at *m/z* 290, 155, 137 and 109 , which indicated that the non-pyrrolobenzodiazepine portion of the lixivaptan was not metabolized (Table 7). The presence of a product ion at *m/z* 96 indicated oxidation at the pyrrole-CH_2- side of the pyrrolobenzodiazepine. 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.

Metabolite M3. The LC/MS mass spectrum of M3 displayed a $[M+H]^+$ at *m/z* 490, 16 Da heavier than lixivaptan, which suggested addition of one oxygen atom to the parent molecule. The 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). M3 was identified as the N-[3-chloro-4-(3-oxo-5,10,11,11a-dihydro-3H-benzo[e] pyrrolo[1,2- a][1,4]diazepine-10-

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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 larger than lixivaptan, suggesting the addition of one oxygen atom to lixivaptan. The LC-MS/MS spectrum of $[M+H]^+$ showed the 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 retention time, UV spectra and mass spectra of M6 were similar to those of an authentic standard, N-[3-chloro-4-(3-oxo-2,3 dihydro 1H,5H benzo[3]pyrrolo [1,2a][1,4]diazepine-10-carbonyl)-phenyl]-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 the parent molecule. The LC-MS/MS spectrum of *m/z* 506 showed fragment ions at *m/z* 462, 290, 155, 137, 132 and 109 (Table 7). The fragment at *m/z* 462, loss of 44 Da, suggested the presence of an aromatic carboxylic acid. The other fragments at *m/z* 290, 155, 137, 132 and 109 were identical to those of lixivaptan, which indicated the modification at the pyrrolobenzodiazepine moiety. Synthetic 1-(2-[2-chloro-4-(5-fluoro-2-methyl-benzoylamino) 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.

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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 carbonylchloroaniline and carbonylfluoromethylbenzene moieties were unchanged and the pyrrolobenzodiazepine moiety was the site of metabolism (Table 7). The product ion at *m/z* 80 indicated 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 H_2O from $[MH\text{-methylenepyrrole}]^+$ (*m/z* 411, not observed) via fragmentation in the products of $[M+H]^+$ 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 M8 showed the $[M+H]^+$ at *m/z* 490 which was16 Da heavier than lixivaptan and suggested a single oxidation of lixivaptan. The MS/MS spectrum

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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 all major human metabolites (≥10% of total plasma radioactivity) have adequate coverage in these species (FDA, 2008; Robinson and Jacobs,2009; ICH, 2009).). The other reason for conducting radiolabeled ADME studies in toxicology species is also to understand the species-dependent metabolism. For example, efavirenz, a potent nonnucleoside reverse transcriptase inhibitor widely prescribed for the treatment of HIV infection, produces renal tubular epithelial cell necrosis in rats but not in monkeys or humans. The radiolabeled ADME studies provide 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 [^{14}C]lixivaptan. The exposures of human metabolites were compared to 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 single dose study and

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compared with the exposures obtained from a 7day 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 [^{14}C]lixivaptan-derived radioactivity in these species. A separate study in the bile duct cannulated rats further suggested that the 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 unabsorbed drug. The absorption of lixivaptan was rapid in both preclinical species and humans, as 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 carried out 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 figure 1. The major metabolites were the pyrrolocarboxylic (M5) and anthranilic (M6) acids of lixivaptan, with the latter having a more prolonged systemic presence. While M6 was a major circulating metabolite in all species studied, M5 was very minor in the rat and dog. The pyrrolinone metabolite (M3) was observed as a major metabolite in rats and dogs, but its significance is unclear in humans due to interference with another incompletely resolved metabolites. Most of the characterized metabolites were formed by single or multiple oxidations

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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 (unpublished data). None of the metabolites that have been tested (M3, M4, M5 and M6) have shown any significant pharmacologic activity (Molinari, 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 which helps simulating their exposures (C_{max} and AUC) at steady-state and eliminates 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_{tlast}) of three metabolites in humans appear to be >10% of total radioactivity exposure. Two most abundant metabolites (M5 and M6) appear to be >100% of parent AUC. Quantitation of M3 was not possible since 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 parent AUC). Based on single dose radiolabeled ADME studies, the exposure of three human metabolites (M6, M7 and M8) was higher at least in one preclinical species (Table 5 and Figure 4). In addition, the combined exposure of metabolites (M1, M2 and M3) was greater in both rats and dogs compared to humans (Table 5 and Figure 4). Exposure of two less abundant metabolites in preclinical species could not be determined. The exposures of

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three metabolites (M3, M5 and M6) estimated from cold studies in humans and preclinical toxicology species using LC-MS/MS method also confirmed that the human metabolites that exceed >10% of total radioactivity have adequate coverage in toxicology species. An undentified peak and M4 were present <5% of total circulating radioactivity (Table 5) and therefore, they are not of 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 M3 metabolite suggested that M3 is just a minor drug-derived product in plasma at steady-state. The amount of M3 after a single dose in ^{14}C-ADME study was overestimated because of its co-elution with other drug-derived material. Therefore, it is very important to develop an adequate separation (HPLC or UHPLC) method which can resolve all metabolites. Recent developments in separation technology, and specifically UHPLC 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

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exposure in at least one of the toxicology species and in most cases 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 $t_{1/2}$ of each metabolite, 2) the base line HPLC separation of each metabolite for the quantitation by radioactivity measurements and there is no induction and/or time dependent inhibition of metabolism.

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Contribution	Author Name
Participated in research design	Chandra Prakash, Zhaoyang Li, Cesare Orlandi, Lewis Klunk
Conducted experiments	Zhaoyang Li
Contributed to new reagents or analytical tools	Chandra Prakash, Zhaoyang Li
Performed data analysis	Chandra Prakash, Zhaoyang Li
Wrote or contributed to the writing of the manuscript	Chandra Prakash, Zhaoyang Li, Cesare Orlandi and Lewis Klunk

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Footnotes

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Figure Legends

Fig. 1. Metabolic pathways of lixivaptan in rats, dogs and humans

Fig. 2. Mean (±SD) plasma concentrations-time curves of lixivaptan and total radioactivity in humans.

Fig. 3. Mean(±SD) plasma concentrations-time curves of lixivaptan and total radioactivity in (A) rat and (B) dog

Fig. 4. HPC-radiochromatograms of circulating metabolites of lixivaptan in humans fter administration of single oral dose of [^{14}C]lixivaptan

Fig. 5. Exposures of lixivaptan and its metabolites in rats, dogs and humans after administration of single oral dose of [^{14}C]lixivaptan

Fig. 6. Mean (±SD) concentration–time curves for the lixivaptan and its major metabolites in human after administration of single oral dose of [^{14}C]lixivaptan

Fig. 7. MS/MS spectra of lixivaptan (*m/z* 474; ^{35}Cl *and m/z* 476, ^{37}Cl)

Table 1. Percent Dose Recovered in the Excreta of Humans, Rats and Dogs

Animal	Dose (mg/kg)	Percent of Radioactive Dose				
		Urine	Bile	Feces	Others*	Total
Human	1	20.5±4.1	NA	63.7±7.9	NA	84.2±8.6
Male Rats	10	5.77±0.25	NA	91.0±4.4	2.20±3.5	99.0±1.2
Female rats	10	7.60±1.4	NA	92.0±3.4	3.51±6.0	103±3.6
Male Rats	10	ND	65.4±6.5	ND	ND	65.4±6.5
Female rats	10	ND	70.5±10.3	ND	ND	70.5±10.3
Male Dog	10	7.95±3.4	NA	83.1±4.4	4.90±3.4	95.9±6.1

*Includes cage wash/debris

Table 2. Pharmacokinetic Parameters (Mean±SD) of Lixivaptan and Total Radioactivity in Humans, Rats And Dogs After A Single Oral Dose of [^{14}C]Lixivaptan*

Analyte	Gender	Dose	T_{max} (h)	C_{max}** ng/ml	$t_{½}$ (h)	$AUC_{(0-t)}$** ng.h/ml	$AUC_{(0-\infty)}$** ng.h/ml
Radioactivity	Human	100 mg	1.2±0.4	1770±590	25.5±7.29	24700±5860	26200±6060
Lixivaptan	Human	100 mg	0.7±0.3	437±120	9.77±3.13	1540±177	1570±177
Radioactivity	Male Rats	10 mg/kg	3.2±0.8	2820±320	36.6±3.6	65700±18700	67100±19500
	Female Rats	10 mg/kg	1.4±0.5	1960±220	33.6±4.7	36700±5000	37200±5100
Lixivaptan	Male Rats	10 mg/kg	0.8±0.6	91±46	1.3±0.2	140±64	219±55
	Female Rats	10 mg/kg	0.8±0.3	338±80	4.2±3.4	1530±640	1890±840
Radioactivity	Male Dogs	10 mg/kg	1.8±0.8	3570±780	69.7±14.9	82800±17200	94900±19900
Lixivaptan	Male Dogs	10 mg/kg	0.8±0.4	759±218	8.8±2.1	4700±1440	4870±1420

* Concentrations of lixivaptan were determined by a validated LC-MS/MS assay

** Cmax and AUC values for total radioactivity are expressed as ng equiv/ml and ng equiv.h/ml, respectively.

Table 3. Relative Abundance (Mean±SD) of Circulating Metabolites in Humans

Metabolite	Ret. Time (min)	Percent of Plasma Radioactivity						
		0.5 h	2 h	4 h	8 h	12 h	24 h	36 h
Parent	34.3	43.5±7.2	13.4±2.3	8.0±2.2	6.7±4.2	5.6±2.4	5.5±4.5	3.6±3.4
M1, M2, M3	<18	31.3±7.7	40.3±7.4	40.3±5.7	37.3±5.2	42±7.8	32.3±5.3	30.9±7.8
Unknown	18.2	3.6±1.7	3.9±1.5	3.0±1.1	1.6±0.5	1.2±0.5	3.0±0.4	
M4	20.2	0.6±0.5	1.5±0.8	2.5±1.0	2.4±1.1	2.1±0.5	2.1±0.4	
M5	23.3	4.1±1.9	16.5±4.4	16.8±2.1	12.2±1.3	11.2±5.0	11±5.1	7.6±2.4
M6	24.5	1.5±0.8	12.6±2.3	19.6±4.4	28.4±7.9	30.1±4.3	38.8±12.5	35.2±3.2
M7	25.2	2.7±0.5	2.2±0.5	2.4±1.3	2.1±0.6	1.6±0.7	4.2±1.9	3.8±1.2
M8	26.3	4.5±1.1	3.7±1.2	3.1±0.1	1.6±0.5	2.4±1.0	0.8±0.2	

Table 4. Relative Abundance (Mean±SD) of Circulating Metabolites Rats and Dogs

Metabolite	Percent of Plasma Radioactivity								
	Male Rats			Female Rats			Dogs		
	1 h	8 h	24 h	1 h	8 h	24 h	2 h	8 h	24 h
Parent	4.1±0.4			29.9±4.2	12.2±2.2		21.2±6.9	14.9±6.7	7.4±8.6
Unknown*	1.6±0.2	2.4±0.3		2.0±0.2	4.3±0.5		1.1±2.2	26.3±2.1	42.9±12.8
M1	7.0±1.3			4.2±1.6	2.9±5.0		13.7±3.8	4.7±3.5	1.4±2.7
M2	3.0±0.5			4.5±1.1	2.1±1.8		8.2±1.4	2.7±3.3	1.6±3.1
M3	5.4±1.5			36.2±2.4	25.2±8.3	2.6±4.6	19.4±3.1	11.1±4.0	3.9±4.5
M5	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
M6	58.5±0.4	73.9±2.0	67.8±3.1	9.0±0.8	42.4±12.3	97.4±4.6	15.3±5.3	29.7±7.2	38.7±11.7
M7, M8	2.8±0.6			4.5±0.5	2.8±2.5		15.6±2.3	7.7±1.9	1.1±2.1

NQ; not quantified
*Eluted close to void volume

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Table 5. Exposure of Lixivaptan and Metabolites in Human, Dog and Rat After Administration of A Single Oral Dose of [^{14}C]Lixivaptan

	Exposure ($AUC_{0\text{-}24\ h}$) of metabolites (ng equiv.h/ml)			
Metabolite	Human	Dog	Male rat	Female Rat
Parent	1860±305	11200	632	5580
M1+M2+M3	8100±2340	13070	832	7960
Unidentified	406±63	ND	ND	ND
M4	268±128	NQ	ND	ND
M5	2680±562	NQ	NQ	NQ
M6	5180±1830	22400	32100	11100
M7+M8	652±153	5640	432	1050

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Table 6. Calculated Exposure (Mean±SD) of Lixivaptan and Metabolites in Humans*

Analyte	^{14}C-Human AME (SD)		Simulated from ^{14}C- study		Cold clinical study*	
	C_{max} (ng/ml)	AUC (ng.h/ml)	Css (ng/ml)	AUCss (ng.h/ml)	Css (ng/ml)	AUCss (ng.h/ml)
Lixivaptan	454±114	1860±305	575±170	2440±1000	514±220	2960±1060
M3**	716±317	8100±2470	942±349	10700±2450	112±42.9	416±167
M5	290±99	2680±591	346±108	3230±589	454±137	3040±991
M6	278±103	5180±2410	428±160	8110±4020	658±201	6610±2110

* subject dosed a single 100 mg dose of lixivaptan for 7 days; exposure at day 7
** quantified with M1 and M2 in ^{14}C-human AME study

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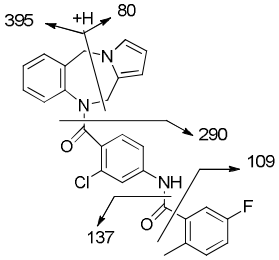
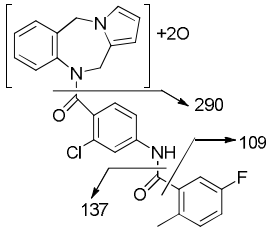
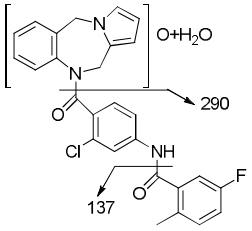
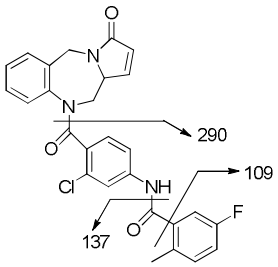
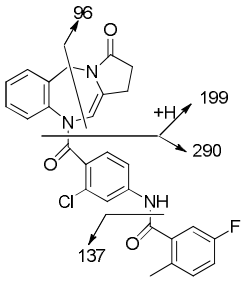
Table 7. Assignment and Proposed Structures of Metabolites of Lixivaptan

Metabolite	MH^+	Structure	Major Fragment Ions
Lixivaptan	490		395,290, 185, 183, 155, 137, 109, 80
M1	506		290, 155, 137, 109, 96
M2	508		290, 155, 137
M3	490		290, 155,137, 109
M4	490		290. 199, 155, 137

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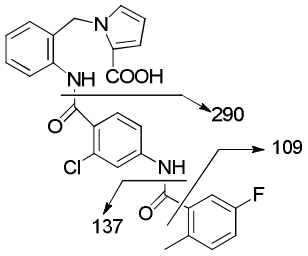
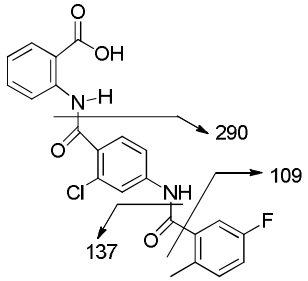
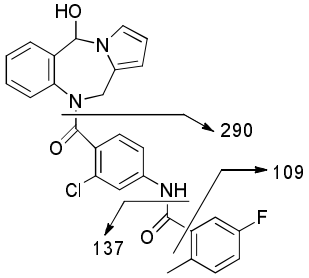
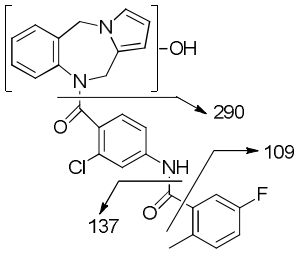
M5	506		290, 155, 137, 109
M6	427		290, 155, 137, 109
M7	490		290, 137, 80
M8	490		183, 137, 80

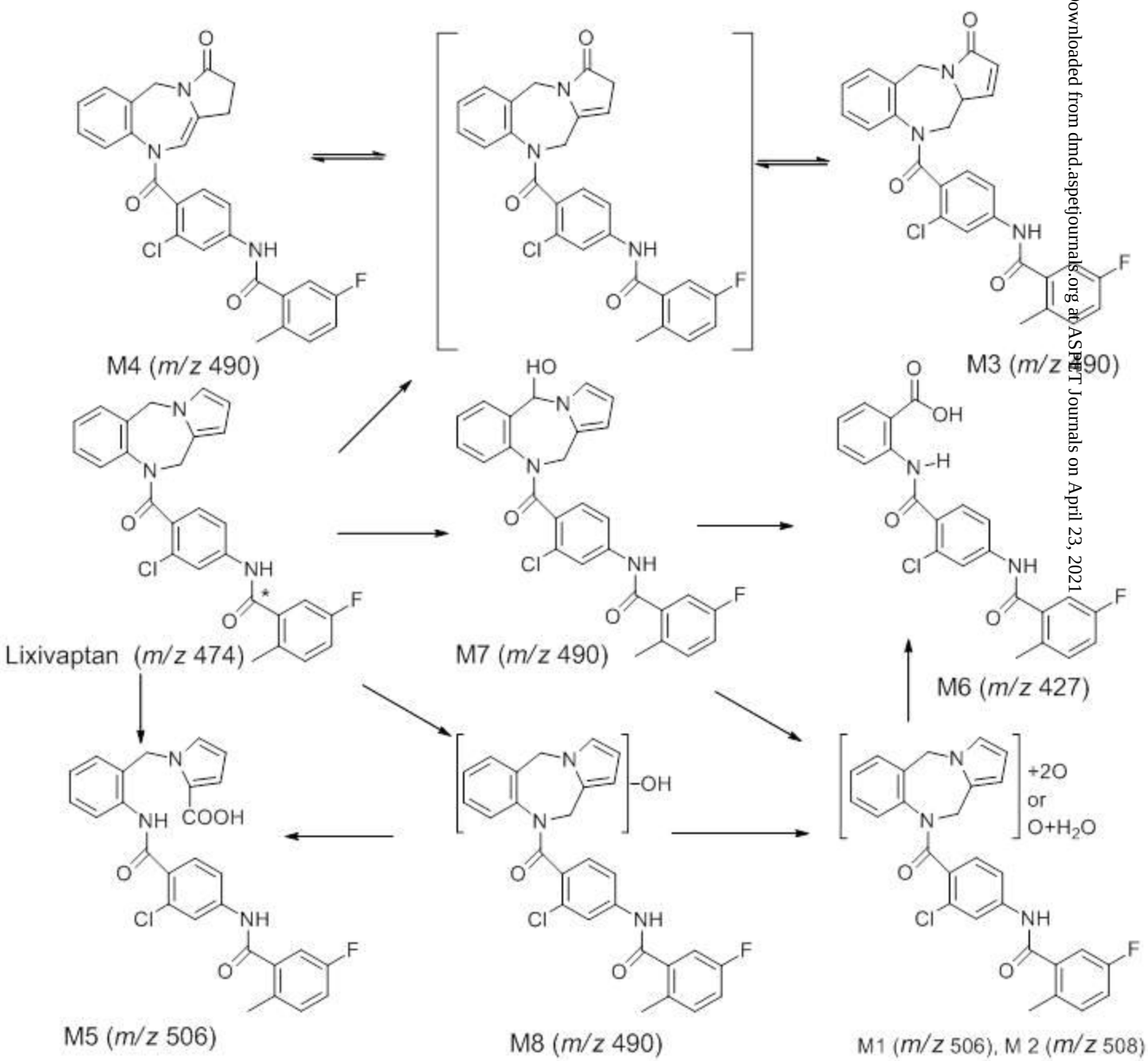


Fig. 1

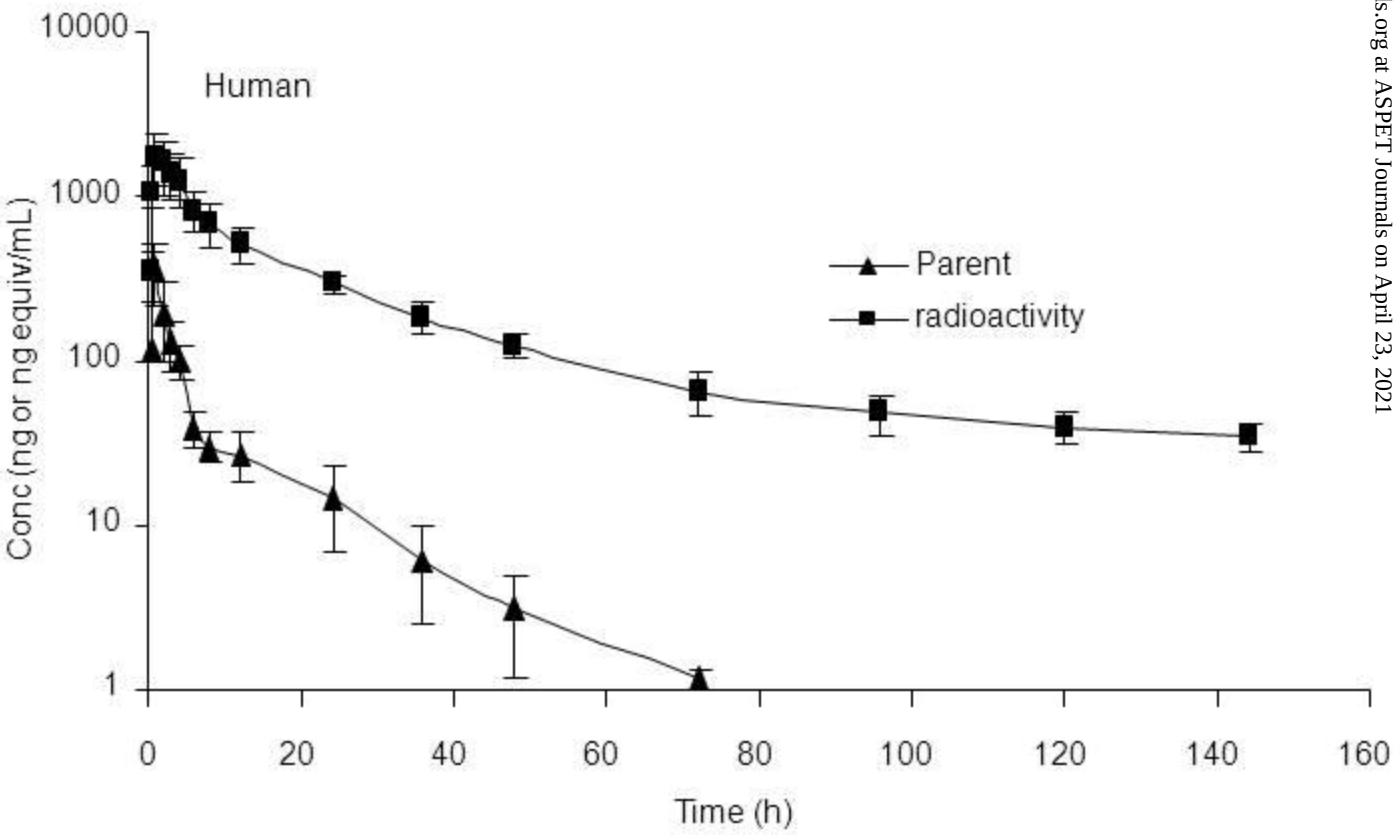


Fig. 2

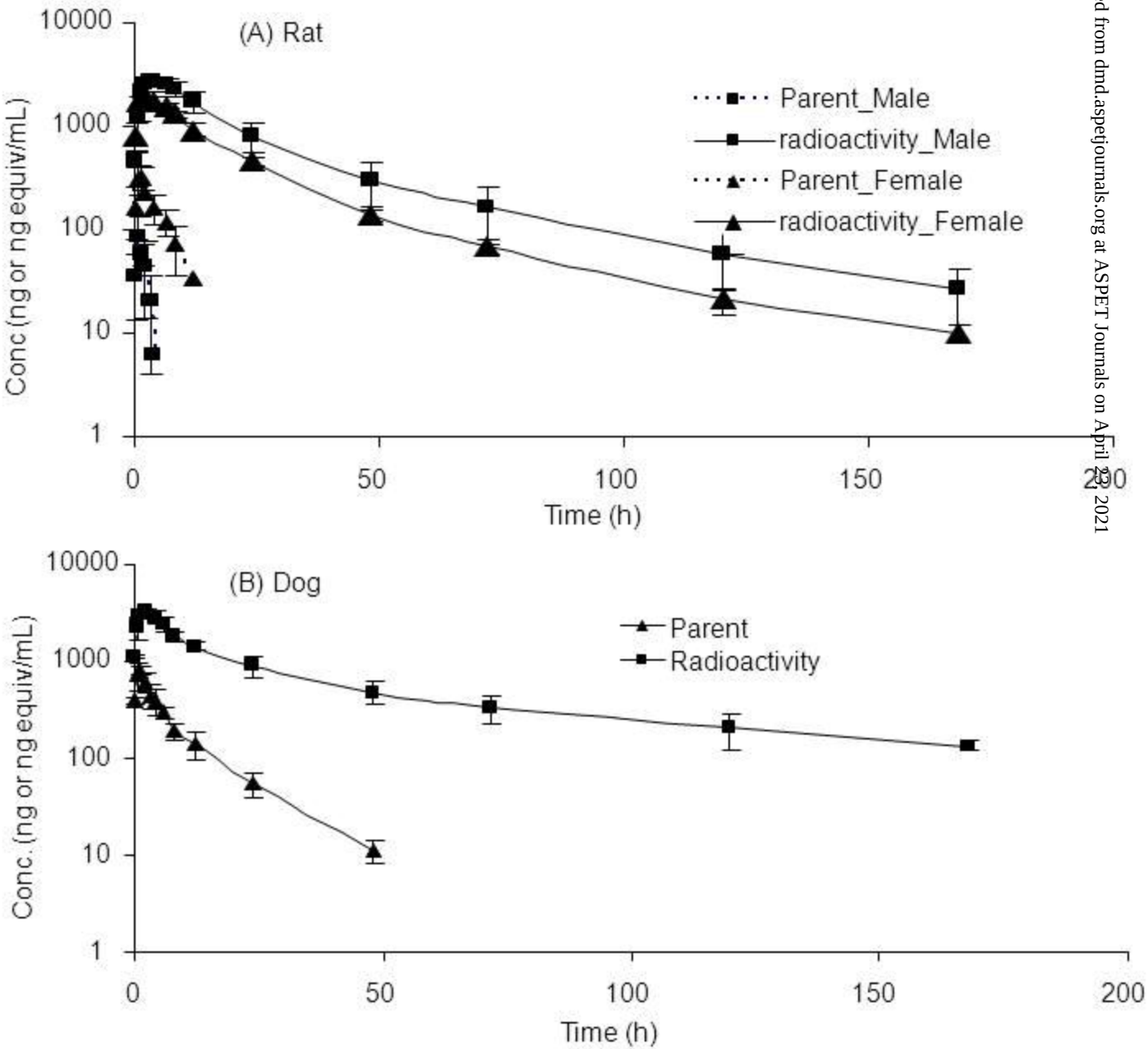
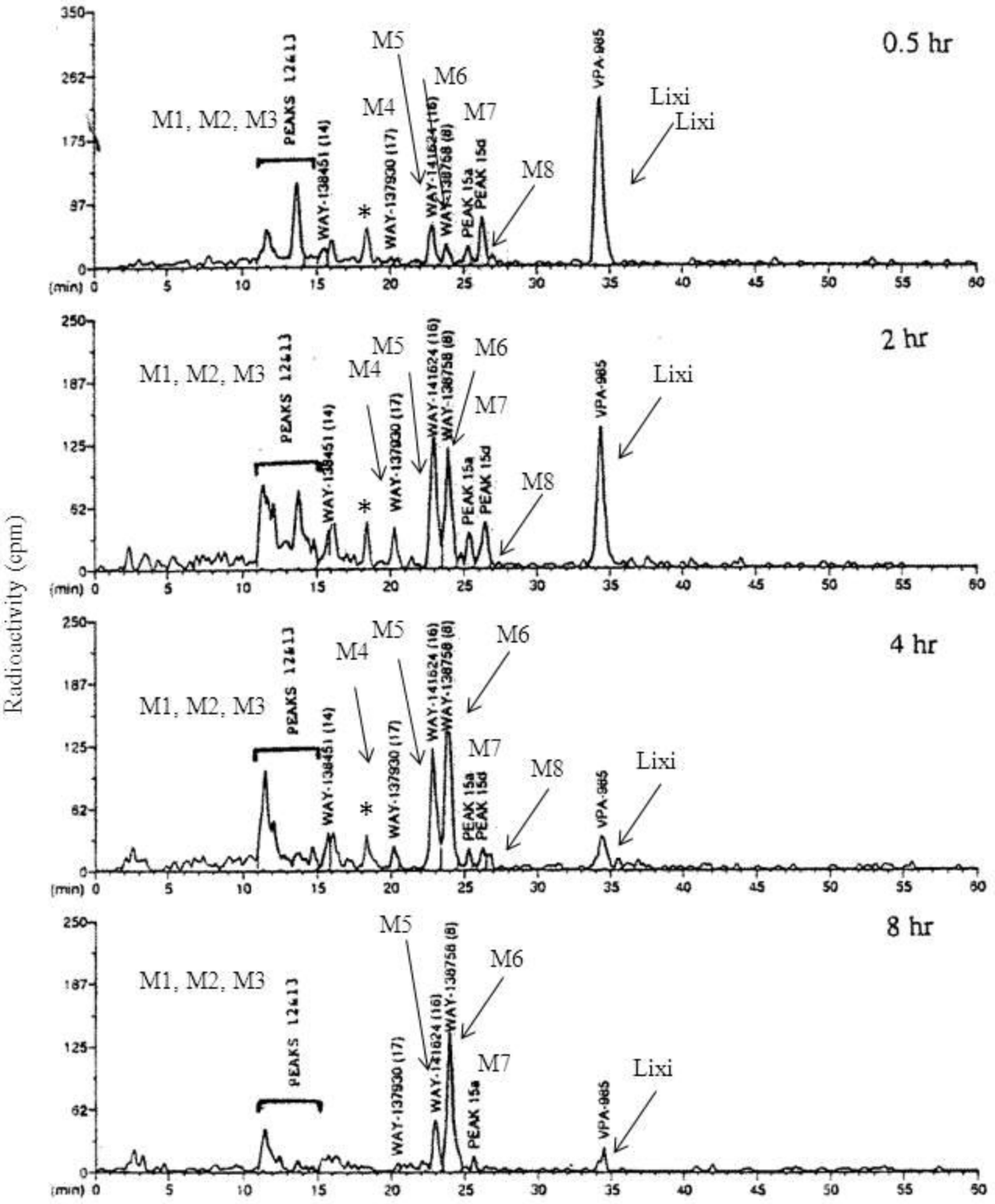


Fig. 3



* unidentified

Fig. 4

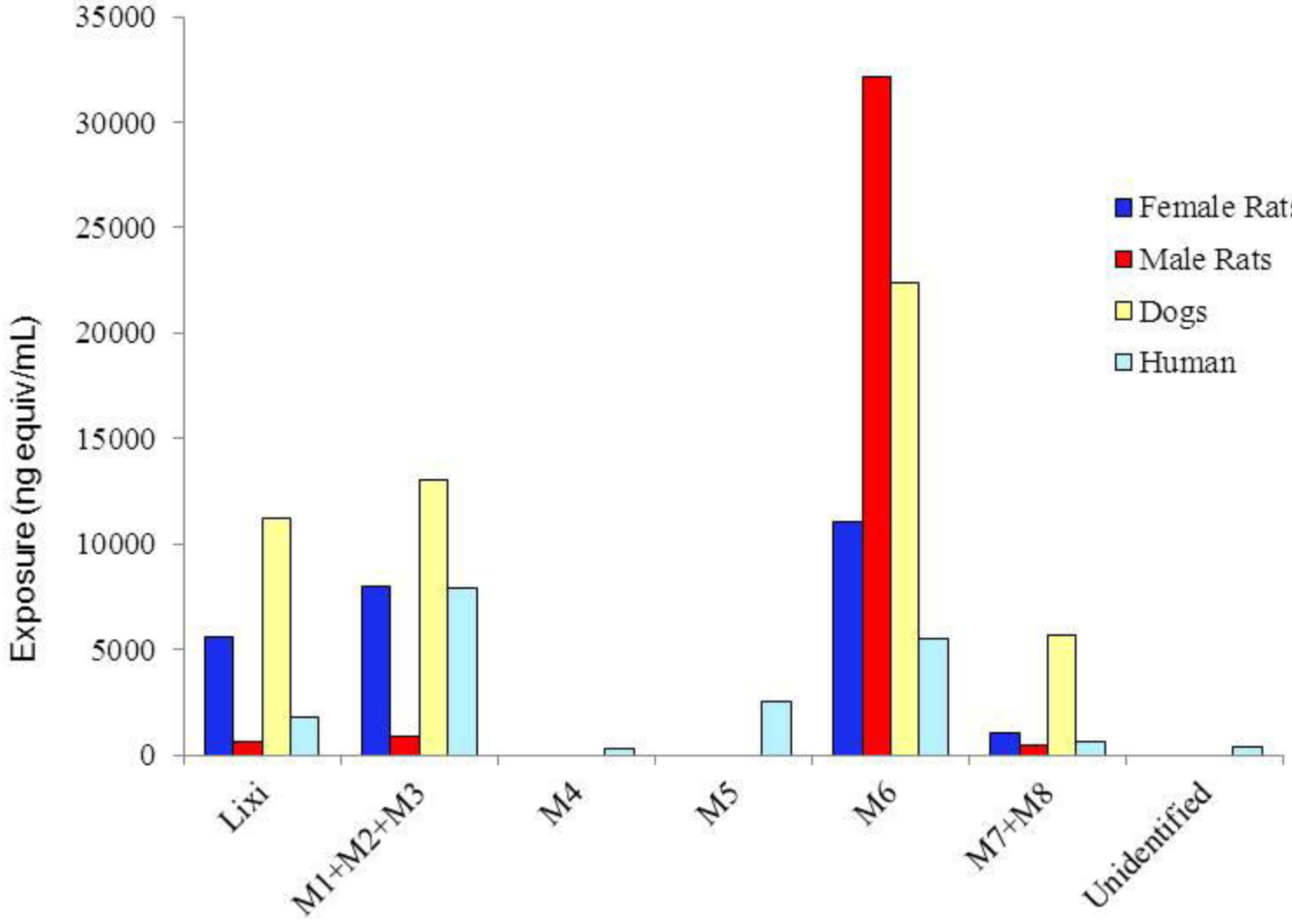


Fig. 5

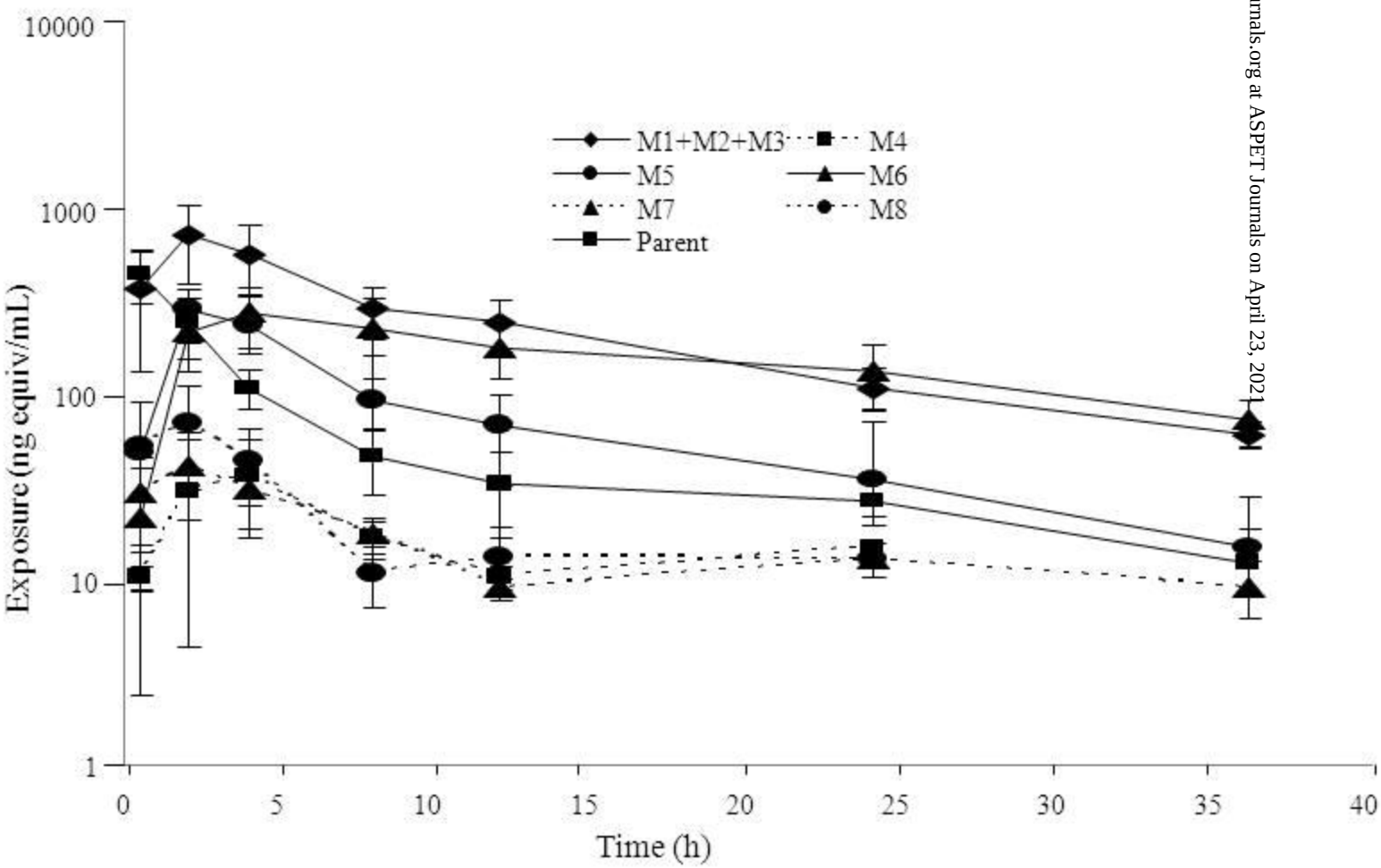


Fig. 6

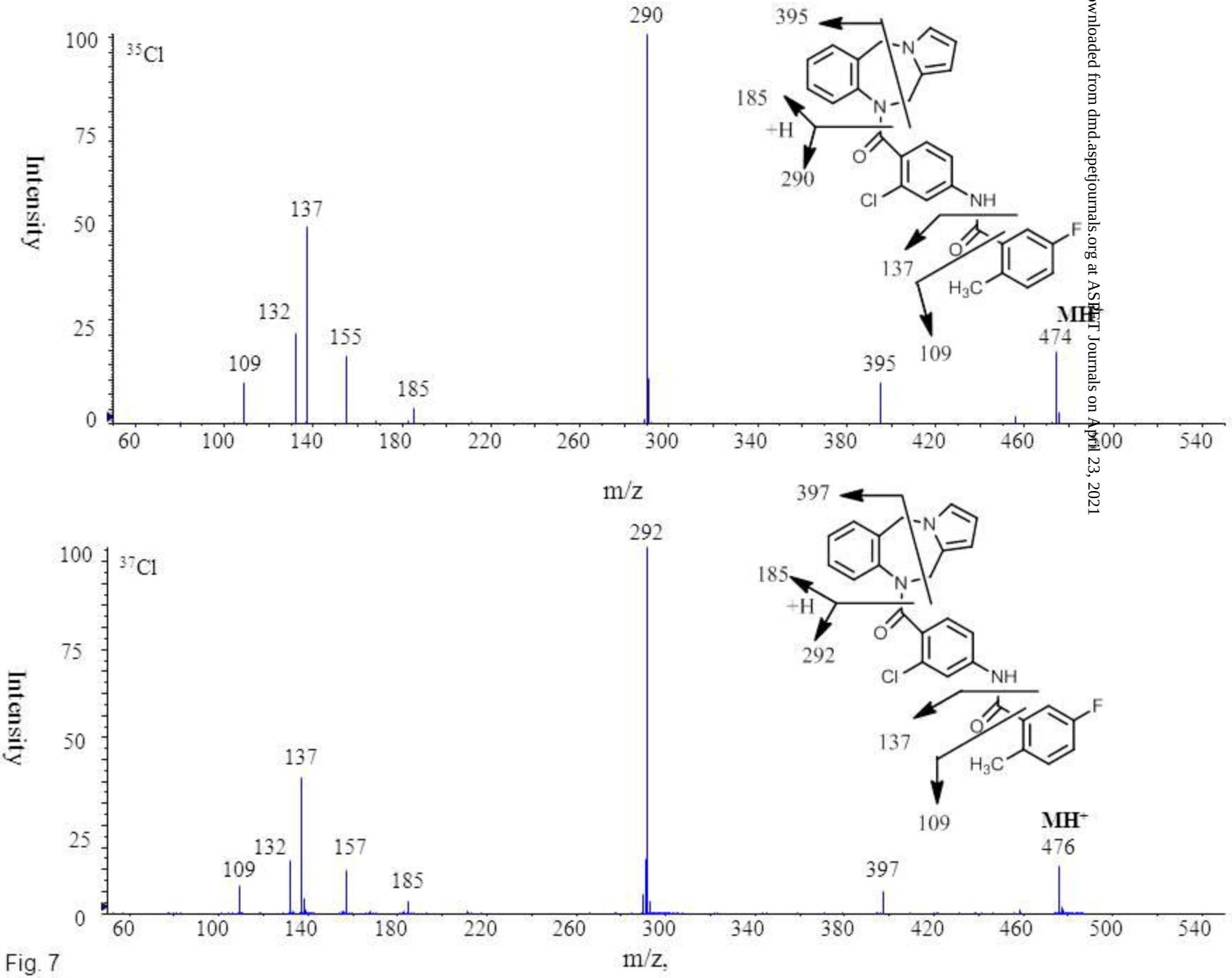


Fig. 7