Pharmacokinetics, Metabolism, and Excretion of [14C]Axitinib, a Vascular Endothelial Growth Factor Receptor Tyrosine Kinase Inhibitor, in Humans

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Received December 16, 2013; accepted March 7, 2014

ABSTRACT

The disposition of a single oral dose of 5 mg (100 μCi) of [14C]axitinib was investigated in fasted healthy human subjects (N = 8). Axitinib was rapidly absorbed, with a median plasma T\text{max} of 2.2 hours and a geometric mean C\text{max} and half-life of 20.2 ng/mL and 10.6 hours, respectively. The plasma total radioactivity-time profile was similar to that of axitinib but the AUC was greater, suggesting the presence of metabolites. The major metabolites in human plasma (0–12 hours), identified as axitinib N-glucuronide (M7) and axitinib sulfoxide (M12), were pharmacologically inactive, and with axitinib comprised 50.4%, 16.2%, and 22.5% of the radioactivity, respectively. In excreta, the majority of radioactivity was recovered in most subjects by 48 hours postdose. The median radioactivity excreted in urine, feces, and total recovery was 22.7%, 37.0%, and 59.7%, respectively. The recovery from feces was variable across subjects (range, 2.5%–60.2%). The metabolites identified in urine were M5 (carboxylic acid), M12 (sulfoxide), M7 (N-glucuronide), M9 (sulfoxide/N-oxide), and M8a (methylhydroxy glucuronide), accounting for 5.7%, 3.5%, 2.6%, 1.7%, and 1.3% of the dose, respectively. The drug-related products identified in feces were unchanged axitinib, M14/15 (mono-oxidation/sulfone), M12a (epoxide), and an unidentified metabolite, comprising 12%, 5.7%, 5.1%, and 5.0% of the dose, respectively. The proposed mechanism to form M5 involved a carbon-carbon bond cleavage via M12a, followed by rearrangement to a ketone intermediate and subsequent Baeyer-Villiger rearrangement, possibly through a peroxide intermediate. In summary, the study characterized axitinib metabolites in circulation and primary elimination pathways of the drug, which were mainly oxidative in nature.

Introduction

Axitinib (AG-013736) (Fig. 1) is an oral medication that has been approved in the United States and multiple countries as a treatment of renal cancer. In the United States, axitinib is indicated for the treatment of advanced renal cell carcinoma in patients who have failed one prior systemic therapy (Inlyta, 2012). Axitinib is an inhibitor of ATP binding to the kinase domain of vascular endothelial growth factor receptors (VEGFRs) 1, 2, and 3 with subnanomolar affinity (Bender et al., 2004; Hu-Lowe et al., 2008; McTigue et al., 2012). The VEGFR signaling network is a regulator of angiogenesis, which is necessary to supply tumors with oxygen and nutrients during phases of rapid growth (Folkman et al., 1971). Solid tumors in many organ systems have high expression of VEGFR compared with normal tissues (Hanahan and Folkman, 1996).

During phase 1 clinical trials the pharmacokinetics of axitinib were evaluated over a dose range of 2–20 mg twice daily in cancer patients (Rugo et al., 2005). Time to maximal plasma concentration (T\text{max}) and half-life (T\text{1/2}) ranged from 1.7 to 6.0 hours and 1.7 to 4.8 hours, respectively. Exposure increased linearly as assessed by C\text{max} and area under the plasma concentration–time curve (AUC). In vitro studies suggested that CYP3A was responsible for substrate consumption of axitinib in human liver microsomes (Zientek et al., 2010). The role of CYP3A in the metabolism of axitinib was supported by results from clinical drug interaction studies where ketoconazole increased the axitinib plasma AUC ratio relative to a control arm by 2-fold and rifampin pretreatment decreased this ratio to 0.2 (Pithavala et al., 2010, 2012b).

Human radiolabeled mass balance studies are usually performed during drug development to identify and quantify the exposure to
metabolites circulating in plasma, elucidate metabolite structures, determine the primary clearance mechanisms of the drug, understand the rate and route of excretion of parent drug and its metabolites, and validate the species used in safety studies. These studies generate important knowledge regarding the disposition of the drug in humans. Furthermore, the studies are typically conducted during drug development as part of drug registration packages and are seldom repeated due to the cost, complexity, and challenges of performing human studies with radioactivity. During the conduct of human absorption, distribution, metabolism, and excretion (ADME) studies, challenges may arise that could compromise the ability to achieve the study objectives defined above. The human ADME study of axitinib, conducted during early clinical development, is an example of a study that advanced our knowledge of the disposition of the drug but had the limitation of low and variable recovery of radioactivity. Herein we describe the knowledge gained during the conduct of the radiolabeled mass balance and metabolite structure elucidation of a single oral 5-mg dose of \(^{14}C\)axitinib to healthy volunteers.

Materials and Methods

Axitinib was synthesized by Pfizer Global Research and Development, La Jolla Laboratories (San Diego, CA). \(^{14}C\)Axitinib was prepared by Pfizer Global Research and Development Radiosynthesis Group (Groton, CT) using the same synthetic route as used for unlabeled axitinib (Borchardt et al., 2006). This route yielded 125 mg of \(^{14}C\)axitinib with a specific activity of 58.3 mCi/mmol, and the position of the incorporation of \(^{14}C\) is shown in Fig. 1. The resulting \(^{14}C\)axitinib was mixed with 811 mg of nonradiolabeled axitinib and subjected to polymer control (crystal form IV) (Campeta et al., 2010), yielding 800 mg of \(^{14}C\)axitinib drug substance mixture with a specific activity of 7.1 mCi/mmol. The purity of \(^{14}C\)axitinib drug substance was 99% by high-performance liquid chromatography (HPLC). Reference standards for M9 (axitinib sulfoxide/\(N\)-oxide), M12 (axitinib sulfoxide), and M15 (axitinib sulfone) were prepared biosynthetically using microorganisms and isolated by HPLC and the structures confirmed by liquid chromatography–mass spectrometry (LC-MS) and NMR (Supplemental Material). M7 (axitinib N-glucuronide) was isolated from urine from this study and characterized by NMR.

Study Design, Dosing, and Sample Collection

This was a single-dose, open-label study conducted at a single center in eight healthy male nonsmoker subjects (six Caucasian, two African American) with a median age of 34 years (range, 25–46 years) and median weight of 83 kg (range, 79–96 kg) who met inclusion/exclusion criteria. The study was conducted in accordance with the Declaration of Helsinki, approved by an institutional review board, and all subjects provided voluntary informed consent. All subjects received a single 5-mg oral dose of axitinib containing 100 \(\mu\)Ci \(^{14}C\)axitinib supplied as a powder for reconstitution. On the day of dosing, 20 ml of cranberry juice was added to each plastic container containing \(^{14}C\)axitinib powder. The container was capped tightly and then shaken vigorously by hand 10 to 12 times until the powder was suspended in the cranberry juice. Subjects drank the resulting suspension directly from the plastic container. The container was then rinsed four times using 20 ml of additional cranberry juice each time. At each rinse, the container was shaken vigorously. Subjects immediately drank the rinsed solutions directly from the container. After drinking the final rinse solution, the subjects swallowed at least 200 ml of tepid water. The oral cavity of each subject was examined after dosing to ensure that all study medication was swallowed. After each drug administration, the empty dosing container was capped and retained for assay of residual radioactivity and, if necessary, for residual drug. Radioactivity determinations of the dose solution and residual remaining in the container confirmed that all subjects received >98% of the expected radioactive dose. Recovery calculations were based on the actual amount of radioactivity administered. Blood samples (7 ml) were collected in K$_2$-EDTA-containing glass Vacutainer tubes prior to dosing and at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 12, 24, 36, 48, 72, 96, 120, 144, and 168 hours after dosing. Additional blood volume (20 ml) was collected at 1, 4, 8, and 12 hours after dosing for metabolite profiling and identification. Following collection of blood samples, plasma was prepared by centrifugation for drug and metabolite analyses and stored frozen. Urine was collected over 4-hour intervals through 12 hours, 12-hour intervals through 48 hours, and every 24 hours thereafter. Feces were collected over 24-hour intervals. Per protocol, excreta samples were collected and tested daily until <1% of the administered dose was recovered, at which point the subject could be discharged from the study. Samples were stored frozen prior to analysis. Precautions were taken during sample collection, processing, and analysis to protect all samples from exposure to visible light.

Measurement of Radioactivity

Plasma (0.25 ml) and urine (1 ml) aliquots were added to Ultima Gold (Perkin Elmer, Waltham, MA) scintillation fluid to a final volume of 20 ml for radioactivity determination by liquid scintillation counting, in triplicate and duplicate, respectively. The concentration of the radioactivity in the whole-blood and red blood cell samples containing potassium EDTA was determined by oxidizing triplicate 0.25-ml aliquots of well mixed whole blood as described below for fecal samples. To each fecal collection was added an equal weight of water followed by homogenization. Three aliquots of each fecal homogenate (0.25–0.47 g) were burned in a Packard 307A oxidizer (Perkin Elmer), which captured the evolved CO$_2$ from each aliquot into Carbosorb (10 ml; Perkin Elmer). Permafluor (10 ml; Perkin Elmer) was added by the oxidizer into each 20-ml scintillation vial. Whole blood (0.25 ml) was processed similarly using the sample oxidizer. All radioactivity measurements were determined using a Tricarb 2100 counter for 20 minutes or a period of time that yielded a counting precision of 95%. Only values >2 times the background were reported. The dpm were converted to nanogram equivalents (ng-eq) per milliliter for each matrix using the known specific activity. For urine and feces, the data were also converted to the percentage of dose for each time interval.

Determination of Axitinib in Plasma and Urine

Axitinib was determined in human plasma and urine by liquid chromatography–tandem mass spectrometry (LC-MS/MS) with plasma methods as described previously (Rugo et al., 2005; Pithavala et al., 2012b). The analysis of axitinib in urine was similar to that described for plasma with the exception of glass tubes instead of the use of a 96-well plate for extraction. These methods employed sample extraction by an ethyl acetate/hexanes liquid-liquid extraction followed by evaporation, reconstitution, and final extract analyses by LC-MS/MS. The methods were validated for the analysis of axitinib over a concentration range of 0.100–25.0 ng/ml using a 200-µl extraction volume for plasma samples and 1.00–500 ng/ml using a 100-µl extraction volume for urine samples. Accuracy, evaluated by percent bias of quality control samples, ranged between –2.0% and 4.0% for plasma and –0.5% and 1.3% for urine across concentrations and analytical runs. Precision, evaluated by CV percentage of quality control samples, ranged from 3.8% to 7.9% for plasma and 1.9% to 3.5% for urine across concentrations and analytical runs.
Calculation of Pharmacokinetic Parameters

Plasma pharmacokinetic parameters [AUC from time 0 to infinity (AUCinf), Cmax, Tmax, and fbio] were estimated by using WinNonlin software, Version 3.2 (Pharsight Corp., Mountain View, CA), and summarized with descriptive statistics as appropriate using the Statistical Analysis Systems software, Version 8.2 (SAS Institute, Cary, NC). All other calculations, including the estimation of urinary, fecal, and total recovery, were also performed using the Statistical Analysis Systems software. As outlined in the study protocol, no formal statistical analyses were performed for this human mass balance study conducted in eight healthy volunteers.

Sample Preparation for Plasma, Urine, and Fecal Extracts for Metabolite Profiling

Plasma samples collected at 0, 1, 4, 8, and 12 hours postdosing from each subject were pooled based on the algorithm described by Hamilton et al. (1981) to generate a plasma pool (7.2 ml). Plasma proteins were precipitated by the addition of four volumes of acetonitrile/methanol [1:1 (v/v)], vortexed, and centrifuged. The supernatant was removed and retained. The pellet was re-extracted as above, and the two supernatants were combined and transferred into glass tubes for evaporation to dryness under N2 at 40°C. The residues were reconstituted in 1 ml of 30:70 (v/v) methanol/water/ammonium acetate (pH 4), and the solutions were transferred into autosampler vials for analysis. The injection volume was 900 μl.

Urine samples collected over prespecified time intervals postdosing from each subject were pooled on a percent weight basis to generate a urine pool (2 ml). After centrifugation, each pooled urine sample was transferred into autosampler vials for analysis. The injection volume was 900 μl.

The fecal homogenates selected for metabolite profiling varied by subject to cover time intervals where the majority of the radioactivity was excreted. The selected fecal homogenates from each subject were pooled on a percent weight basis to generate a homogenate pool (2 ml). Each pooled sample was precipitated by the addition of four volumes of acetonitrile, vortexed, and centrifuged. The supernatant was removed and retained. The pellet was re-extracted as above, and the supernatants were combined and transferred into glass tubes for evaporation to dryness under N2 at 40°C. The residues were reconstituted in 1 ml of 30:70 (v/v) methanol/water/ammonium acetate (pH 4), and the solutions were transferred into autosampler vials for analysis. The injection volume was 800 μl.

To investigate causes of observed variable fecal recovery of radioactivity in this study, the fecal homogenization procedure was also put to additional scrutiny. In an effort to ascertain if an alternate homogenization procedure might produce better recovery of radioactivity from the fecal samples, a series of experiments were undertaken. Briefly, the following alternate procedures were evaluated using blank fecal samples as well as actual fecal samples from this study: 1) manual vigorous shaking of the fecal homogenate slurry for 1 to 2 minutes before sampling for radioactivity counting; 2) sonication of the fecal homogenate slurry after manual vigorous shaking and before sampling for radioactivity counting; 3) liquid extraction of the axitinib-related material from the fecal homogenate slurry (using an acetonitrile-acidified water system that is routinely used for solubilizing axitinib), involving three successive extractions of the fecal homogenate, followed by the pooled supernatant and extraction pellet being counted for radioactivity; and 4) evaluation of any radioactivity adsorbed to the polyethylene bag used for the homogenization by oxidation of two cut sections of the polyethylene bag that held the fecal homogenate.

Metabolic Profiling and Structure Elucidation

All metabolite profiling and structure elucidation were performed using HPLC coupled in-line with radioactivity monitor (β-RAM) and MS detection with electrospray ionization (ESI) source in positive mode. The instrumental components were as follows: Agilent 1100 HPLC Pumps/Autosampler (Agilent Technologies, Wilmington, DE), 4.0 × 3.0 mm guard column C18 (Phenomenex, Torrance, CA), Cosmosil 5PYE column 150 × 4.6 mm (Waters, Milford, MA), LCQ-Deca XP Ion Trap MS (ThermoFinnigan, San Jose, CA), Model C ARC StopFlow System (AIM Research Company, Newark, DE), Model 3 β-RAM RadioDetector (500-μl cell) (INUS Systems, Tampa, FL), and StopFlow AD Scintillation Cocktail (AIM Research Company). The mobile phase consisted of 20 mM ammonium acetate in water adjusted to pH 4.0 with acetic acid (solvent A) and acetonitrile (solvent B). The flow rate was maintained at 1.0 ml/min.

Axitinib and metabolites were eluted using a linear gradient in mobile-phase composition summarized as follows: 100% solvent A for 10 minutes, a linear gradient to 50% solvent A/50% solvent B at 60 minutes, a linear gradient to 100% solvent A at 61 minutes, and held at 100% solvent A to 70 minutes. The HPLC effluent was split so that 20% of the flow was introduced into the mass spectrometer via the ESI source while 80% was diverted to the β-RAM detector. The analog outputs from the ARCβ-RAM and MS detectors were recorded in real time by the ARC data system Version 2.4 (AIM Research Company). The major operating parameters for the ion-trap ESI/MS method were as follows: positive ionization mode, spray voltage (5.0 kV), capillary voltage (5.0 V), tube lens offset (55 V), capillary temperature (200°C), sheath gas flow rate (80, arbitrary), auxiliary gas flow rate (20, arbitrary); and negative ionization mode, spray voltage (5.0 kV), capillary voltage (–47.0 V), tube lens offset (–60 V), capillary temperature (200°C), sheath gas flow rate (80, arbitrary), auxiliary gas flow rate (20, arbitrary). LC-MS spectra were acquired over a mass range of 200–1400 m/z for all samples. Mass spectra were processed using either Xcalibur Version 1.4 (ThermoFinnigan) or the ARC data system. Radiochromatographic data were processed using the ARC data system only. Metabolite screening was performed to manually search for molecular ions representing possible metabolites. Ion-trap experiments were performed to generate multistage mass spectra for the selected molecular ions representing possible metabolites of axitinib. Helium at a constant pressure of 40 psi was used as the damping and collision gas for all MS experiments. The mobile phase, gradient, guard cartridge, column, and source conditions used were identical to the ion-trap LC-ESI/MS method. Precursor ion isolation window, activation amplitude, activation Q, and activation time were set at 1.5 amu, 30%–50%, 0.25 millisecond, and 30 millisecond, respectively. The chemical structures of the axitinib metabolites were proposed based on metabolite prediction and mass spectral interpretation. Definitive structures (Wilmad-LabGlass, Vineland, NJ) were assigned for isolated metabolites using NMR spectroscopy or by comparison with authentic synthetic standards.

Metabolite Biosynthesis and Isolation and NMR Spectral Characterization

Isolation of Carboxylic Acid Metabolite (M5) and N-Glucuronide of Axitinib (M7). Metabolites M5 and M7 were isolated from human urine. Briefly, pooled urine samples from each subject were combined to generate a mixed urine pool and treated with acetonitrile. Following vortexing and centrifugation of the mixture, the supernatant was separated, concentrated, and further lyophilized. The residue was reconstituted in 1 ml of mobile phase and injected onto a 900-μl sample loop and separated by HPLC using the same analytical column and the gradient system used for profiling the metabolites. The fractions containing the glucuronide conjugate were collected (detection of the metabolite was done by UV signal) and evaporated under a steady stream of N2. The structures of M5 and M7 were characterized by 1H NMR. Metabolite M7 was also investigated using one-dimensional (1D) nuclear Overhauser effect (nOe) spectroscopy. Approximately 3 μg of the isolated M7 was dissolved into 120 μl of dimethylsulfoxide-d6 (DMSO-d6; Cambridge Isotope Laboratories, Inc., Tewksbury, MA) and transferred to a 3-mm NMR tube (Wilmad 355-PP; Wilmad-LabGlass). The sample of axitinib was prepared by dissolving 51 mg into 0.5 ml of DMSO-d6 and transferring the material to a 5-mm NMR tube (Wilmad 355-PP; Wilmad-LabGlass). NMR measurements were made on a Bruker Avance DRX spectrometer (Bruker Corporation, Billerica, MA) operating at 500.13 MHz. 1H frequency at 30°C (rt 0.01) using a 5-mm TXI Zgradient CryoProbe (Bruker Corporation). The M7 1H NMR spectrum was acquired with 4000 scans and 32,768 complex data points using a spectral width of 10,000 Hz and an acquisition time of 1.64 seconds. An additional relaxation delay of 1.0 second was added between pulses to allow for T1 relaxation. NMR data for axitinib was acquired under the same conditions, with the exception that only 16 scans were needed. For the M7 nOe data, 20,000 scans were acquired using a 1D nOe experiment and selectively inverting the gluconic acid anomeric proton resonance. Spectra were acquired and processed using Bruker’s XWINNMR software, version 3.5-patch level 6.

Preparation of Metabolites M9, M12, and M15. Authentic metabolite standards of M9, M12, and M15 were prepared either using microbes or chemically using meta-chloroperbenzoic acid (MCPBA) in support of definitive structure determination. For microbial synthesis, 40 fungi and 40 bacteria, the majority obtained from the American Type Culture Collection (Manassas, VA).
and a few from in-house sources (Pfizer Inc., Groton, CT), were screened for M12 formation. The bacteria and fungi were grown in 20 g glucose, 5 g soy flour, 5 g yeast extract, 5 g K2HPO4, 5 g NaCl, and 1 g MgSO4·7H2O, adjusted to a final volume of 1 liter of water, adjusted to pH 7.2, and sterilized. All strains were grown at 28°C and 250 rpm on a rotary shaker. After 2 and 3 days, 0.2 mg of axitinib from a 10% ethanol solution was added. After 3 and 6 days, the reactions were analyzed by LC-MS using a Cosmosil SPYF column (150 × 4.6 mm) with a mobile phase of acetonitrile/50 mM ammonium acetate (pH 4.5), at a flow rate of 1.0 ml/min and UV detection at 254 nm. For positive-mode MS detection, the mobile phase consisted of 20 mM ammonium acetate in water adjusted to pH 4.0 with acetic acid and acetonitrile. Among all strains screened, Cunninghamella echinulata and Streptomyces griseus showed the highest oxygenation activity. M12 was produced in abundance of >80%, and this was used to prepare a large batch of the metabolite.

For preparative transformation, C. echinulata obtained from the American Type Culture Collection was grown from an agar plate into a 100-ml preculture using the screening medium and conditions. After 2 days, 10 ml of the preculture was inoculated into 1 liter of culture on a 4-liter shake flask (× 5 liters). The culture was grown for 24 hours, and then axitinib was added (0.2 g/l substrate for each 1 liter of culture). Oxidation was followed by reverse-phase HPLC, and the reaction was stopped after 8 days. The mycelium was removed from the culture by filtration, and the oxidation products were extracted three times with one volume of chloroform each. After removal of CHCl3 in vacuo, crude product (~500 mg) was obtained. The crude product was purified by silica gel flash chromatography, using CH2Cl2/acetone/MeOH (10:1:1) as eluent to afford 30 mg of the sulfoxide M12. For preparative transformation of axitinib to M12 by S. griseus, the microbe was grown from an agar plate into a 100-ml preculture using the screening medium and conditions. After 2 days, 10 ml of the preculture was inoculated into 1 liter of culture in a 4-liter shake flask (× 10 liters). The culture was grown for 24 hours, and axitinib was added (0.1 g/l substrate for each 1 liter of culture). Oxidation was followed by reverse-phase HPLC and the reaction was stopped after 8 days. The mycelium was removed from the culture by filtration, and the oxidation products were extracted three times with one volume of chloroform each. After removal of CHCl3 in vacuo, the crude product (1.5 g) was obtained. The crude product was purified by silica gel flash chromatography using CH2Cl2/acetone/MeOH (10:1:1) as eluent to afford 80 mg of sulfoxide and yielded 8–9 mg pure sulfoxide.

Preparative Transformation of Axitinib to M15 by MCPBA. MCPBA was added to 200 mg of axitinib suspended in 10 ml of methylene chloride at 0°C. The reaction mixture was stirred at room temperature for 7 hours. After removal of solvent, the crude products were purified by silica gel chromatography using methylene chloride/acetone/methanol (10:1:1) as eluent to afford M15 (63 mg, 30%) and M9 (11 mg, 5%). The structures of M9, M12, and M15 were elucidated using 1H and 13C NMR methods by dissolving the compounds into DMSO-d6. The chromatographic profiles and 1H and 13C NMRs of M9, M12, and M15 are presented in Supplemental Figs. S1–S7.

Evaluation of M7 and M12 for Inhibition of Kinases and hERG. Axitinib metabolites M7 (glucuronide) and M12 (sulfoxide) were evaluated in cellular assays for their ability to inhibit kinase activities according to the methods described in Hu-Loewe et al. (2008). M12 was tested for inhibition of vascular endothelial growth factor (VEGF)-stimulated human vascular endothelial cell (HUVEC) survival, platelet-derived growth factor receptor-β [enzymelinked immunosorbent assay (ELISA)], basic fibroblast growth factor–stimulated HUVEC survival, and KIT (ELISA), while M7 (glucuronide) was evaluated for inhibition of VEGFR2 (ELISA) in transfected porcine aorta endothelial cells. M7 and M12 were also evaluated for the inhibition of human ether-a-go-go-related gene (hERG) to assess their risk to prolong cardiac QTc interval. M7 was evaluated for inhibition of IKr using whole-cell voltage-clamp electrophysiology, and M12 was evaluated for inhibition of ligand-binding fluorescence polarization according to methods described by Deacon et al. (2007).

Results
Pharmacokinetics of Axitinib Radioactive Equivalents and Axitinib in Plasma

The mean plasma concentration–time profile for total radioactivity and axitinib parent drug following the administration of 5 mg of [14C]axitinib to healthy human subjects is presented in Fig. 2, and the derived pharmacokinetic parameters are presented in Table 1. Total radioactivity and parent axitinib reached maximal concentration in plasma at ~2–3 hours postdose. The mean axitinib plasma Cmax and AUC was about 32% and 15% of total radioactivity, suggesting that total circulating axitinib metabolites exceeded the parent drug. The terminal elimination half-life after oral dosing was similar for axitinib and total radioactivity, suggesting similar parent and total metabolite elimination rates from the body. The total [14C]axitinib-derived radioactivity whole-blood exposure, assessed by Cmax and AUC, was about half of the plasma values, indicating that the combination of parent and metabolite was preferentially distributed in plasma (data not shown).

Excretion of Axitinib Radioactive Equivalents in Urine and Feces

The urine and feces were collected for 144 hours and up to 288 hours postdose from eight healthy male subjects following an oral 5-mg dose of radiolabeled axitinib. The excretion of total radioactivity in urine and feces in each subject is shown in Fig. 2. The recovery of total radioactivity was lower than expected, especially in subjects 1001 and 1003, and variable between subjects. The total recovery was 37.9% and 16.0% in subjects 1001 and 1003, respectively. In the remaining six subjects, the overall recovery of radioactivity ranged from 51.3% to 77.9% of the administered dose. The median recovery (all subjects) was 59.7%. In all but one subject (1001) with low recovery, the excretion of radioactivity in feces was greater than in urine. The median recovery across all subjects in feces and urine was 37.0% and 22.7%, respectively. Urinary recovery was consistent across the eight subjects; however, fecal recovery was quite variable (2.5%–60%). An investigation into potential causes of the poor recovery was conducted. This included confirmation of the administered radioactivity in the dose and additional homogenization and sampling of fecal homogenates. In subjects 1002 and 1003, a change in bowel habits was noted, with no fecal sample obtained for several consecutive collection intervals (Fig. 3). Fecal samples were homogenized and resampled for radioactivity determination in three randomly selected subjects; however, no difference was noted from the original counts (data not shown).

Axitinib Plasma Metabolites

Plasma samples collected at 1, 4, 8, and 12 hours were combined according to the pooling method of Hamilton et al. (1981) to create a 0–12-hour plasma pool for each subject. A representative radiochromatogram is presented in Fig. 4. These samples were profiled by
HPLC, and the individual percentage of each axitinib-derived component is presented in Table 2. The mean percentages of axitinib and related metabolites present in circulation were as follows: M7, 50.4%; M12, 16.2%; and axitinib, 22.5%; with a coefficient of variation of <27% for each component.

**Axitinib Urinary and Fecal Metabolites**

Also shown in Table 2 are the percentages of the dose excreted in the urine and feces for each metabolite, and representative radiochromatograms are shown in Fig. 4. The mean percent metabolite excreted in urine was largest for M5 (5.7%), followed by M12 (3.5%), M7 (2.6%), M9 (1.7%), and M8a (1.3%), while unchanged axitinib was not detectable. In addition, a more sensitive LC-MS assay was used to detect and quantify lower concentrations of unchanged axitinib in urine. In five out of eight subjects, there was no detectable axitinib (<1 ng/ml) in any urine sample. In the remaining three subjects, low concentrations of axitinib were noted (≥3.1 ng/ml) in urine samples collected from 0–12 hours. This independently confirmed the results of radiochemical profiling that there was no appreciable renal clearance of unchanged axitinib in urine. In fecal extracts the primary component was unchanged axitinib (12.0%), followed by roughly equal amounts of M14/15 (5.7%), M12a (5.1%), and an unidentified metabolite (5.0%).

**Identification of Axitinib Metabolites**

Axitinib gave a molecular ion at m/z 387 ([M+H⁺]) in a positive ion mode. The mass spectrum of m/z 387 gave characteristic major fragment ions at m/z 356, 222, and 166 (Table 3). The fragment ion at m/z 356 was a result of the loss of the methylamine moiety from axitinib and subsequent formation of acylium ion, whereas the fragment ions at m/z 222 and 166 were obtained from the cleavage of the sulfide linkage. The structures (confirmed or proposed) of metabolites of axitinib are shown in Fig. 5, and Table 3 depicts the molecular and major fragment ions of all the detected metabolites.

**Metabolite M5.** Metabolite M5 showed a protonated molecular ion at m/z 387 in the full scan. The mass spectrum of m/z 387 showed major fragment ions at m/z 342 and 218 (Table 3). The main fragment ion at m/z 342 gave characteristic major fragment ions of axitinib, showing a loss of 176 amu from m/z 387 ([M+H⁺] of axitinib). An even molecular ion of the metabolite suggested a loss of nitrogen from the molecule. The fragment ions at m/z 311 (Table 3) were consistent with a loss of a methylamine moiety from m/z 342, while the fragment ion at m/z 166 suggested a cleavage of the sulfide moiety. Both the loss of the methylamine group and the fragment ion of m/z 166 were observed in the mass spectral fragmentation of axitinib. Other fragment ions at m/z 296 and 265 showed a loss of 46 amu from m/z 342 and 311, respectively (Table 3). The loss of 46 amu, which is characteristic of the presence of a carboxylic acid group in the molecule (Ling et al., 2006; Dalvie et al., 2010), suggested that M5 was a carboxylic acid derivative (Table 3). The structure of the metabolite was further confirmed by 1H NMR after isolating the metabolite from human urine. The 1H NMR spectrum of M5 revealed a loss of the pyridine ring and the vinyl protons observed in axitinib (Table 4; the 1H NMR spectra of axitinib and M5 are shown in Supplemental Figs. S8 and S9, respectively). This was replaced by a singlet due to methylene protons at 3.8 ppm, sandwiched between the carboxy group and the indazole ring. The metabolite was therefore proposed as 2-(6-(2-(methylcarbamoyl)phenyl)thio)-1H-indazol-3-yl)acetate acid (Fig. 5).

**Metabolite M7.** Metabolite M7 showed a protonated molecular ion at m/z 563 in the full scan and was consistent with addition of 176 amu to axitinib and suggested that M7 was a glucuronide conjugate of axitinib. The mass spectrum of m/z 563 gave fragment ions at m/z 387 (the molecular ion of axitinib) and 356 (Table 3). The ion at m/z 387 indicated a loss of 176 amu from m/z 563 and corresponded to the molecular ion of axitinib. The fragment ion at m/z 356 suggested a loss of the methylamine moiety from m/z 387 as observed in the mass spectrum of axitinib as described above. The exact site of glucuronidation could not be discerned from the mass spectral details. The site of glucuronidation was evaluated further by isolating the metabolite from human urine and subjecting it to NMR analysis. 1H NMR of M7 showed all the assigned resonances of axitinib in addition to the resonance signals from protons in the glucuronic acid moiety (Table 4; the 1H NMR is shown in Supplemental Figs. S10, A and B). The key change in the 1H NMR spectrum of M7 was the shift of the resonance signal at 7.65 ppm for the proton at the 7-position of the indazole ring to 7.95 ppm (Table 4) and suggested attachment of the glucuronic acid moiety to the indazole nitrogen. The point of attachment of the glucuronic acid moiety to the indazole nitrogen was confirmed with additional 1D nOe measurements. A selective nOe to the nearby proton at the 7-position was observed (Fig. 6) upon selective inversion of resonance of the anomic proton of glucuronic acid.

**Metabolite M8a.** Metabolite M8a gave a signal at m/z 579 suggesting an addition of 192 amu to the molecular ion of axitinib. A mass spectrum of M8a at m/z 579 showed major fragment ions at m/z 403, 385, 373, and 356 (Table 3). The molecular ion at m/z 403 (loss of 176 amu) in the mass spectrum suggested that the metabolite was a glucuronide conjugate of an oxidative metabolite of axitinib. The modification was possibly on the N-methyl amide moiety since the mass spectrum also showed a fragment ion at m/z 356, which was consistent with the loss of the methylamine group in axitinib. The fragment ion at m/z 356 and 373 suggested a loss of water and a hydroxymethyl moiety from m/z 403, respectively, further confirming that the methyl group on the methylamine moiety was the site of hydroxylation. Although the exact site of glucuronidation could not be discerned from the fragment ions, the possible site of glucuronidation was either the nitrogen of the indazole moiety as observed in M7 or the hydroxyl group of the hydroxymethyl amide moiety.

---

**TABLE 1**

<table>
<thead>
<tr>
<th>Axitinib</th>
<th>Total Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tmax</td>
<td>Cmax</td>
</tr>
<tr>
<td>h</td>
<td>µg/ml</td>
</tr>
<tr>
<td>Geometric mean</td>
<td>2.22</td>
</tr>
<tr>
<td>Upper 90% CI</td>
<td>2.72</td>
</tr>
<tr>
<td>Lower 90% CI</td>
<td>1.81</td>
</tr>
</tbody>
</table>

CL confidence interval.
Metabolite M9. Metabolite M9 showed a protonated molecular ion at \( m/z \) 419 in the full scan, which was 32 amu greater than axitinib. This indicated that M9 was a dioxygenated axitinib metabolite. Its mass spectrum at \( m/z \) 419 gave major fragment ions at \( m/z \) 388, 324, and 296 (Table 3). The fragment ion at \( m/z \) 388 indicated a loss of 31 amu, which was consistent with the loss observed in the fragmentation of axitinib, while the fragment ions at \( m/z \) 324 and 296 resulted from a loss of 64 amu from \( m/z \) 388 and a subsequent loss of 28 amu from \( m/z \) 324, similar to the losses observed in the spectrum of M15 (as described below). Although the loss of 64 amu generally indicates
extrusion of sulfur dioxide and therefore the presence of a sulfone, the
different retention times of M9 to M15 suggested that M9 was a different
dioxygenated metabolite of axitinib. The regiochemistry of oxygenation
could not be discerned from the mass spectral data. Comparison of
retention time of M9 with \textit{N}-oxide derivative of axitinib sulfoxide,
prepared using MCPBA, suggested that the oxidation in M9 occurred on
the sulfur as well as the pyridine nitrogen as shown in Fig. 5.

**Metabolite M12.** Metabolite M12 showed a protonated molecular ion at \textit{m/z} 403, suggesting an addition of 16 amu to 387 ([M+H\(^+\)]\(^\)\) of axitinib). An addition of 16 amu indicated that M12 was a

** Fig. 4.** A typical reverse-phase HPLC radiochromatogram of plasma (0–12 hours), urine (0–36 hours), and fecal extracts (varied time interval) following a 5-mg oral dose of \([^{14}\text{C}]\text{axitinib}\) (100 \(\mu\text{Ci}\)).

**TABLE 2**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Percentage of Metabolite in Radiochromatogram or Dose for Each Individual Subject and as Mean and S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1001</td>
</tr>
<tr>
<td>Plasma (0–12 h)</td>
<td></td>
</tr>
<tr>
<td>M7</td>
<td>59.2</td>
</tr>
<tr>
<td>M12</td>
<td>9.3</td>
</tr>
<tr>
<td>Axitinib</td>
<td>18.9</td>
</tr>
<tr>
<td>Urine (0–36 h)</td>
<td></td>
</tr>
<tr>
<td>M5</td>
<td>4.1</td>
</tr>
<tr>
<td>M7</td>
<td>5.5</td>
</tr>
<tr>
<td>M8a</td>
<td>2.3</td>
</tr>
<tr>
<td>M9</td>
<td>2.1</td>
</tr>
<tr>
<td>M12</td>
<td>6.3</td>
</tr>
<tr>
<td>Feces (varied time interval)</td>
<td></td>
</tr>
<tr>
<td>UNK</td>
<td>0.7</td>
</tr>
<tr>
<td>M12a</td>
<td>0.4</td>
</tr>
<tr>
<td>M14/15</td>
<td>3.1</td>
</tr>
<tr>
<td>Axitinib</td>
<td>6.2</td>
</tr>
</tbody>
</table>

UNK, unidentified metabolite.
### TABLE 3

Mass spectral fragmentation and structures of axitinib and proposed metabolites

<table>
<thead>
<tr>
<th>Structure</th>
<th>[M+H]+</th>
<th>Fragment Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axitinib</td>
<td></td>
<td>387 356 222 166</td>
</tr>
<tr>
<td>M5</td>
<td></td>
<td>342 311 296 265 166</td>
</tr>
<tr>
<td>M7</td>
<td></td>
<td>563 387 356</td>
</tr>
<tr>
<td>M8a</td>
<td></td>
<td>579 403 385 373 356</td>
</tr>
<tr>
<td>M9</td>
<td></td>
<td>419 388 324 296</td>
</tr>
<tr>
<td>M12</td>
<td></td>
<td>403 372 355 344 327</td>
</tr>
<tr>
<td>M12a</td>
<td></td>
<td>403 372 354 344</td>
</tr>
<tr>
<td>M14</td>
<td></td>
<td>403 372 355 344</td>
</tr>
<tr>
<td>M15</td>
<td></td>
<td>419 388 324 296</td>
</tr>
</tbody>
</table>
mono-oxygenated metabolite. The mass spectrum at m/z 403 gave
fragment ions at m/z 372, 355, 344, and 327 (Table 3). The fragment
ion at m/z 372 indicated a loss of 31 amu, which was consistent with
the loss observed in the fragmentation of axitinib. The fragment ion at
m/z 355 resulted from a loss of 48 amu and was characteristic of a loss
of a sulfoxide moiety from the molecule (Bu et al., 2007; Shimizu
et al., 2009). The ions at m/z 344 and 327 indicated a loss of 28 amu
from m/z 372 and 355, respectively, and suggested a loss of either
carbonyl functional groups from the acylium intermediate that was formed from
cleavage of the N-methylamide or loss of nitrogen from the indazole
ring. The metabolite was confirmed by comparing it with the synthetic
standard, which was prepared using microbial reactions.

**Metabolite M12a/M14.** Metabolites M12a and M14 gave a pro-
tonated molecular ion at m/z 403 in the full scan, which suggested that
both the metabolites were formed via mono-oxygenation of axitinib. The mass spectrum of M12a gave major fragment ions at m/z 372, 354, and 344 (Table 3). The presence of the ion at m/z 372 indicated a loss of 31 amu from m/z 403, suggesting a loss of the methylamine moiety, and the ion at m/z 344 was 28 amu lower than m/z 372, corresponding to loss of N2 from the indazole or a carbonyl moiety. These losses were similar to those observed in the fragmentation pattern of axitinib. The fragment ion at m/z 354, on the other hand, indicated a loss of a water molecule from m/z 372, which suggested that hydroxylation was at a site that was susceptible to dehydration and eliminated pyridine or indazole or the phenyl group in the axitinib molecule as probable sites of oxygenation. It was therefore speculated that the site of oxidation was the alkene moiety in the molecule, and the possibility of the metabolite being the epoxide of axitinib could not be ruled out. The proposed mechanism leading to the loss of a water molecule from an epoxide is presented in Fig. 7.

The mass spectrum of M14 at m/z 403 gave major fragment ions at
m/z 372, 355, and 344 (Table 3). As in the case of M12a, the fragment
ions at m/z 372 and 344 indicated that pyridine was the likely site of
modification. The fragment ion at m/z 355 indicated a loss of 17 amu
from m/z 372, which was characteristic of an N-oxide metabolite,
especially the pyridine N-oxide. This suggested that M14 was most
likely a pyridine N-oxide metabolite of axitinib. However, since
neither M12a nor M14 was confirmed by NMR or their respective
synthetic standards, these metabolites have been represented as
Markush structures in Fig. 5 and Table 3.

**Metabolite M15.** Metabolite M15 showed a protonated molecular
ion at m/z 419, suggesting an addition of 32 amu to 387 ([M-H+] of
axitinib). This indicated that M15 was a dioxygenated metabolite. Its
mass spectrum at m/z 419 gave major fragment ions at m/z 388, 324,
and 327 (Table 3). The fragment ion at m/z 388 indicated a loss of 31 amu, which was consistent with the loss observed in the fragmentation of axitinib. The fragment ion at m/z 324 resulted from a loss of 64 amu from m/z 388. Published reports indicate that compounds with a sul-
fone moiety such as the sulfonamide and/or sulfones show a character-
istic loss of 64 amu due to extrusion of sulfur dioxide from the
molecule (Wang et al., 2003; Bu et al., 2007). The fragment ion at m/z
296 indicated a loss of 28 amu from m/z 324 and suggested a loss of
either a carbonyl group from the acylium intermediate that was formed from cleavage of the N-methylamine or loss of nitrogen from the indazole ring similar to that observed in the spectrum of the sulfoxide metabolite (M12). This suggested that M15 was a sulfone metabolite of axitinib. The modification of the sulfide to the sulfone was further confirmed by comparing the spectral properties and the retention time with the synthetic standard that was prepared using MCPBA.

Assessment of Kinase Activity of M7 and M12

Because M7 and M12 were the primary circulating metabolites in human plasma, they were evaluated for biologic activity in relevant assays. The activity of axitinib in the assays used to evaluate the metabolites has been reported previously (Hu-Lowe et al., 2008). M7 (axitinib N-glucuronide) was not suspected to be active because it lacks the indazole NH that forms a critical hydrogen bond in the ATP-binding site of the VEGFR kinase domain (McTigue et al., 2012). The IC<sub>50</sub> for inhibition of VEGFR2 autophosphorylation in porcine aorta endothelial (PAE) cells by M7 was 1990 nM (N = 7), which was 8300-fold higher than axitinib. M12 (axitinib sulfoxide) was evaluated for inhibition of VEGF-stimulated HUVEC survival and a few other related kinase activities. Unlike axitinib, M12 showed minimal activity against VEGF-stimulated HUVEC survival, platelet-derived growth factor receptor-β, and KIT in cell assays, with IC<sub>50</sub> values of greater than 100, 810, and 310 nM, which were greater than 400-, 470-, and 290-fold higher than the IC<sub>50</sub> values for axitinib. The IC<sub>50</sub> for inhibition of basic fibroblast growth factor–stimulated HUVEC survival by M12 was >1000 nM, an assay where axitinib also showed weak activity. These data indicated that M7 and M12 were far less potent against the target and some related kinases, and hence were considered pharmacologically inactive metabolites. M7 and M12 had hERG-inhibitory potencies of >30 μM and >79 μM in cellular voltage-clamp and binding assays, respectively.

Discussion

The objectives of the study were to investigate the disposition of an oral 5-mg dose of [14C]axitinib in healthy human subjects to identify and quantify the exposure to metabolites circulating in plasma, elucidate metabolite structures, determine the primary clearance mechanisms of axitinib, understand the rates and routes of excretion of axitinib and its metabolites from the body, and validate the species used in safety studies. The majority of these study objectives were met, although not without some limitations.

Metabolism was the primary route of axitinib clearance because only 12% of the unchanged drug was observed in the feces and none was detected in the urine. Essentially, five primary metabolites detected in human excreta comprised the key pathways for axitinib clearance: sulfoxide (M12), N-glucuronide (M7), hydroxymethyl (M8a precursor), oxidation products (M12a/M14), and N-oxide (M9). In human plasma, the only metabolites detected were axitinib sulfoxide and axitinib N-glucuronide, and together they comprised about 66.6% of the drug-related plasma AUC. Many of the metabolites in excreta were not detected in plasma. This suggested that the urinary metabolites M5, M8a, and M9 were cleared by the kidney much more rapidly than their formation clearance, preventing...
accumulation to detectable concentrations in blood. Similarly, the fecal metabolites M12a, M14/15, and the unknown metabolite are likely rapidly transported into the bile and are not substrates for hepatic apical transporters that could facilitate their transfer to blood.

Assessment of circulating metabolites in humans contributes to an overall understanding of their potential impact on the safety and efficacy of a drug. From a safety point of view, gathering knowledge of the in vivo metabolic profile of a drug in humans has gained more importance since the publication of position papers on drug metabolites in safety testing as well as regulatory guidance documents on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals (Davis-Bruno and Atrakchi, 2006; European Medicines Agency, 2009; Neddeman et al., 2011). [14C]Axitinib disposition has been studied in the mouse and dog (Smith et al., manuscript in preparation),

Fig. 6. Comparison plot of 1D nOe difference spectrum and normal 1D 1H NMR spectrum of M7.

Fig. 7. Proposed mechanism for the formation of fragment ion m/z 354 from an epoxide metabolite.
and the sulfoxide (M12), the metabolite of primary concern for such assessments, was detected in sufficient concentrations in the plasma of both species when compared with the results from the present study in humans. Further, neither M7 nor M12 was pharmacologically active for VEGFR or other kinases, nor were they found to exert activity in hERG assays. Hence, both metabolites did not warrant further evaluation and quantification in nonclinical studies or clinical trials.

The enzymology of axitinib metabolism has been investigated in human liver microsomes and recombinant enzymes (Zientek et al., 2010). CYP3A4/5 were the major enzymes involved in the oxidative metabolism of axitinib. This was consistent with the results from several studies that investigated the clinical pharmacokinetics of axitinib. When ketoconazole 400 mg was given as an interacting drug with 5 mg axitinib as the object, a 2-fold increase in \( C_{\text{max}} \) and AUC was observed (Pithavala et al., 2012b). Axitinib has a moderate intravenous clearance and an oral bioavailability of 58% (Inlyta, 2012). Thus, a 2-fold interaction with ketoconazole is suggestive that CYP3A is the major clearance mechanism.

The formation of M7 was primarily catalyzed by UGT1A1 (Zientek et al., 2010). Given the polymorphic nature of UGT1A1, several genetic polymorphisms were investigated using a meta-analysis of data from 11 axitinib healthy volunteer clinical studies in 315 subjects to determine if there was association of specific genotypes with the observed variability in axitinib pharmacokinetics (Brennan et al., 2012). However, none of the evaluated genotypes were found to significantly contribute to the observed between-subject variability in axitinib pharmacokinetics. This suggests that the conversion of axitinib to the \( N \)-glucuronide is a minor clearance mechanism even though it is a major metabolite in plasma. The factors that cause the apparent disconnect between metabolite abundance in plasma and clearance are known and have been well described by others (Lutz et al., 2010; Loi et al., 2013). Finally, taken together, these and other data suggest that liver is the primary organ involved in the clearance of axitinib from the body.

The formation of a carboxylic acid metabolite (M5) from axitinib was unusual in that it involves loss of a pyridine ring via a cleavage of a carbon-carbon bond. While the mechanism for its formation is unknown, one possible pathway might involve oxidation of axitinib to an epoxide metabolite, which could undergo rearrangement to a putative ketone intermediate (Fig. 8). Although not observed in any matrices, this intermediate could undergo a carbon-carbon cleavage via a mechanism analogous to the Baeyer-Villiger rearrangement via a peroxy intermediate, which involves insertion of an oxygen atom into the carbon-carbon bond next to a carbonyl group (aldehyde or ketone) to form an ester. Formation of this intermediate could occur through a cytochrome P450-catalyzed Fe(III)-OOH-mediated attack on the carbonyl carbon of the putative ketone metabolite to form an enzyme-bound peracid intermediate (Ortiz de Montellano and DeVoss, 2005). Some evidence suggests that mammalian flavin mono-oxogenases may also catalyze these rearrangements in the metabolism of xenobiotics (Chen et al., 1995; Lai et al., 2011). Hydrolysis of the corresponding ester would result in the carboxylic acid metabolite M5. Radical decomposition of the peroxysmecial intermediate can also lead to M5. A similar Baeyer-Villiger mechanism has been proposed previously by Mayol et al. (1994) in the formation of the triazolodione metabolite of nefazodone and Dalvie et al. (1996) in the formation of a cleaved product of trovafloxacin.

Another important objective of this study was to determine the rates and routes of excretion of \([^{14}C]\)axitinib in humans. In most subjects the majority of the radioactive dose was recovered in the first 48 hours. The median overall recovery in all subjects was 59.7% (16.0%–77.9%), with 22.7% (13.5%–28.2%) in urine and 37.0% (2.5%–60.2%) in feces. When assessing the rates and routes of excretion, the excretion of \([^{14}C]\)axitinib equivalents in the feces was the major route in three subjects, whereas in other subjects fecal excretion was lower and/or the excretion pattern was unusual. The overall recovery of \([^{14}C]\)axitinib was low across subjects, and this result did not meet expectations, initiating an investigation as to the potential causes. We confirmed that all subjects received the entire dose by counting the radioactivity before and after administration. Further, the plasma axitinib \( C_{\text{max}} \) and AUC presented in Table 1 was similar to what has been observed in other studies involving healthy human subjects receiving the same 5-mg single oral dose of the drug (Pithavala et al., 2010, 2012a,b). The recovery of radiolabeled axitinib equivalents in urine was consistent between subjects, and the urinary excretion rate was similar and aligned with the plasma pharmacokinetics of total radioactivity and parent drug. These results indicated that the recovery of radioactivity from feces was variable and warranted further investigation. Several different procedures were evaluated to determine if homogenization of the fecal samples might have been incomplete and thus contributed to the recovery variability. Fecal samples from three subjects were rehomogenized and sampled in triplicate; however, the results did not differ from the original sampling (data not shown). We did note that the fecal output was low in some subjects. In particular, one subject with the lowest fecal excretion of radioactivity only had reported fecal collections on 3 out of 10 days during the study (Fig. 3). Another two subjects also had an unusual profile of generated fecal samples. Total fecal output collected during the first 5 days of the study was lower in the subjects with the lowest recovery (data not shown). Finally, the radiolabel recovery in mouse and dog mass balance studies with \([^{14}C]\)axitinib was >90%, which suggested that the radiolabel was likely incorporated in a metabolically stable position in the molecule (Smith et al., manuscript in preparation), and thus radiolabel instability was an unlikely contributor to recovery variability. The exact reason(s) for incomplete total recovery is unknown, but it was determined that it was not due to dosing errors or suboptimal fecal homogenization techniques and may have to do with irregular and incomplete/sparse bowel movements. Beumer et al. (2006) conducted an extensive review of human mass balance studies for anticancer and other therapeutic agents. They found that the recovery was between 70% and 85%.

In summary, the disposition of 5 mg \([^{14}C]\)axitinib in humans was investigated. While the overall recovery of radioactivity was low, the majority of the study objectives were met. The plasma concentration–time profile of total radioactivity was similar to the parent drug. Only two metabolites were present in circulation, axitinib \( N \)-glucuronide (M7) and axitinib sulfoxide (M12), both pharmacologically inactive. The axitinib human ADME results were consistent with hepatic metabolism being the main clearance mechanism, with excretion of parent drug and metabolites in the feces as the main route of excretion and excretion of axitinib metabolites in the urine as the secondary route. The metabolite structures for the majority of the metabolites were elucidated with a proposed mechanism for the formation of an unusual carboxylic acid metabolite.

**Acknowledgments**

The authors thank Dr. David Hoelscher at PPD Development (Austin, TX) for the conduct of the clinical mass balance study, Andy Marquardt from PPD Development for radioactivity counting, and Adrienne Manning from Charles River Laboratories (Worcester, MA) for the bioanalysis of axitinib in plasma and urine.
Dr. Shanghui Hu from the Pfizer Pharmaceutical Sciences group contributed to the structure elucidation of axitinib N-glucuronide. Dr. Dana Hu-Lowe assessed pharmacologic activity of axitinib metabolites. Maxwell Parker and Dr. Jill Steidl-Nichols evaluated M7 and M12 for hERG interaction. Finally, Dr. Dennis Smith is acknowledged for helpful discussions on the recovery of the radiolabel.

Authorship Contributions

Participated in research design: Bu, Klamerus, Pithavala, Pool, Wu.

Conducted experiments: Bu, Deese, Kang.

Contributed new reagents or analytic tools: Bu, Deese.

Performed data analysis: Bu, Dalvie, Deese, Hee, Kang, Klamerus, Pithavala, Pool.

Wrote or contributed to the writing of the manuscript: Bu, Dalvie, Deese, Kang, Pithavala, Pool, Smith, Wu.

References


Fig. 8. Proposed mechanism for formation of the carboxylic acid metabolite (M5).

Figure S1: HPLC profile of axitinib metabolites by Cunninghamella echinulata.

Figure S2: HPLC profile of axitinib metabolites by *Streptomyces griseus.*

Figure S3: Characterization of the axitinib sulfoxide metabolite M12 by $^{13}$C NMR.

Figure S4: Characterization of the axitinib sulfone metabolite M15 by $^1$H-NMR.
Pharmacokinetics, metabolism and excretion of \(^{14}\text{C}\)axitinib, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in humans. Bill J. Smith, Yazdi Pithavala, Hai-Zhi Bu, Ping Kang, Brian Hee, Alan J. Deese, William F. Pool, Karen J. Klamerus, Ellen Y. Wu and Deepak K. Dalvie. Drug Metabolism and Disposition.

Figure S5: Characterization of the axitinib sulfone metabolite M15 by \(^{13}\text{C}\) NMR.

**Figure S6: Characterization of the axitinib mixed sulfoxide/N-oxide metabolite M9 by ¹H-NMR.**

Figure S7: Characterization of the axitinib mixed sulfoxide/N-oxide metabolite M9 by $^{13}$C-NMR.

Figure S8: $^1$H NMR spectrum of axitinib.

Figure S9: Comparison of the aromatic region (A) and the aliphatic region (B) of $^1H$ NMR spectrum of metabolite M5 with that of axitinib.
Pharmacokinetics, metabolism and excretion of $[^{14}\text{C}]$axitinib, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in humans. Bill J. Smith, Yazdi Pithavala, Hai-Zhi Bu, Ping Kang, Brian Hee, Alan J. Deese, William F. Pool, Karen J. Klamerus, Ellen Y. Wu and Deepak K. Dalvie. Drug Metabolism and Disposition.

Figure S10A: Comparison of the aromatic region of $^1$H NMR spectrum of metabolite M7 with that of axitinib.

Asterisk indicates a downfield shift of the proton signal in the NMR of M7 (top panel) relative to axitinib (bottom panel).

Figure S10B: Comparison of the aromatic region of 1H NMR spectrum of metabolite M7 with that of axitinib.