**Drug Metabolism and Disposition (DMD-AR-2024-001751)**
**Supplemental Material**

# Absorption, Distribution, Metabolism, and Excretion of Icenticaftor (QBW251) in Healthy Male Volunteers at Steady State and In Vitro Phenotyping of Major Metabolites

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## 1. Inclusion and Exclusion Criteria

### Inclusion Criteria

The key inclusion criteria were: male subjects, 18–55 years of age (both inclusive) in good health as determined by medical history, physical examination, vital signs, ECG, and laboratory tests at screening; normal vital signs at screening and baseline (temperature: 35.0–37.5°C; systolic blood pressure: 90–139 mmHg; diastolic blood pressure: 50–89 mmHg; pulse rate: 45–90 bpm); body mass index of 18–30 kg/$m^2$; and body weight of ≥55 to <120 kg.

### Exclusion Criteria

The key exclusion criteria were: exposure to radiation at a dose range of 0.1–1.0 mSv in the past year, exposure to 1.1–2.0 mSv radiation in the past 2 years, or exposure to 2.1–3.0 mSv radiation in the past 3 years; recent use of other investigational drugs (within five half-lives, or within 6 months, or within 30 days after dosing); significant illness not resolved within 2 weeks before initial dosing; recent (within the past 3 years) and/or recurrent history of autonomic dysfunction (e.g., recurrent episodes of fainting, palpitations, and other symptoms); history of multiple and recurring allergies or allergy to the study compound/compound class; history of immunodeficiency diseases; history or presence of clinically significant abnormalities in ECG and PR-interval ≥200 ms, resting QTcF ≥450 ms, and QRS-complex >120 ms; any surgical or medical condition that can significantly alter the absorption, distribution, metabolism, and excretion (ADME) of drugs (e.g., inflammatory bowel disease, ulcer, gastrointestinal or rectal bleeding, or major gastrointestinal surgery); pancreatic injury or pancreatitis, or laboratory values outside the normal range; absence of a regular defecation pattern; consumption of particular citrus fruits (e.g., Seville oranges, grapefruit) and cruciferous vegetables (e.g., Brussels sprouts, broccoli, cabbage, cauliflower) during the past 7 days before dosing; and smoking (use of tobacco/nicotine products in the past 3 months).

## 2. Sample Collection and Preparation

All collections of blood, urine, and feces samples were recorded on the appropriate sample log form. Samples of whole blood, urine, and feces were collected over a 7-day (up to Day 12) collection period after [$^{14}$C]icenticaftor dosing on Day 5 according to the study assessment schedule. Additional samples were to be collected for up to 13 days (up to Day 18) post-dose if the release criteria defined in the protocol were not met. However, if the release criteria were met, then biological samples were collected for up to 9 days (up to Day 14) post-dose.

### Blood and Plasma Samples

All blood samples (8 mL or 18 mL) were collected either by direct venipuncture or using an indwelling cannula inserted in a forearm vein, and a total blood volume of approximately 492 mL was drawn. Plasma and blood concentrations of total radioactivity and icenticaftor were determined at prescheduled time points for up to 216 hours after [$^{14}$C]icenticaftor dosing. Three aliquots (0.3 mL each) were removed for radioactivity determination in blood, after which they were frozen immediately and stored below −20°C. One aliquot of 1 mL was separated for the potential analysis of metabolites, and frozen and stored below −60°C. The remaining blood sample was centrifuged at 4°C to obtain plasma. Three plasma aliquots of 0.25 mL each were removed for radioactivity determination. Two aliquots of 0.4 mL each were reserved for icenticaftor analysis; these samples were frozen and stored below −65°C. The remaining plasma was removed for metabolite analysis, and frozen and stored below −60°C.

Samples for icenticaftor analysis were processed by Veeda Clinical Research Pvt. Ltd. (Gujarat, India) and metabolism samples transported to Novartis Pharma AG (Basel Switzerland) for processing/analysis. All radiometry samples were analyzed by PRA Health Sciences (Bioanalytical Laboratory, Groningen, The Netherlands).

**Urine Samples**

Following the administration of the radiolabeled drug on Day 5, all urine samples were collected during the entire post-dose observation period (0–216 hours) in separate portions at time intervals 0–6, 6–12, 12–24, 24–48, 48–72, 72–96, 96–120, 120–144, 144–168, 168–192, and 192–216 hours post-dose. Urine portions were pooled per subject within any planned collection interval. All portions collected (0–216 hours) were cooled rapidly to below 10ºC (for up to 30 minutes in an ice bath). Urine pH was determined; for a pH below the range of 6.5–7, 5 M sodium hydroxide was added to increase the pH to pH 7. The time period pools were stored in a refrigerator (4–8°C) during the sampling period. At the end of each collection period, the total volume or weight of the collected sample was measured and recorded. Aliquots were separated for radioactivity determination. Two aliquots of 20 mL each were separated for metabolite analysis, frozen and stored below −60°C. The remaining urine sample was retained in the original collection bottles and stored below −60°C.

Metabolism samples were transported to Novartis Pharma AG (Basel Switzerland) for processing/analysis. All radiometry samples were analyzed by PRA Health Sciences (Bioanalytical Laboratory, Groningen, The Netherlands).

**Fecal Samples**

A pre-dose blank fecal sample was collected. Following the administration of the radiolabeled drug on Day 5, all fecal samples were collected during the post-dose sample collection period of 216 hours (in-house phase). Each fecal sample was collected separately in a plastic container. The fecal sample production date and time were recorded. Individual sample containers were stored in a refrigerator at 4–8°C until transfer by the clinical laboratory to PRA Health Sciences (Bioanalytical Laboratory, Groningen, The Netherlands) for processing, aliquoting, and radioactivity determination. Each portion was diluted with one to two volumes of water containing a suspension stabilizer (1% (v:v) carboxymethylcellulose) and homogenized. Aliquots

were separated for radioactivity determination. Two aliquots of 20 g each were separated for metabolite analysis. The remaining fecal samples were reserved for later use if needed. Samples were frozen and stored at −20°C or below −60°C until processing or analysis. Samples for metabolite analysis and reserve samples were shipped to Novartis Pharma AG (Basel, Switzerland).

### 3. Analysis of Total Radioactivity

Liquid scintillation counting (LSC) was used to measure radioactivity in the whole-blood, plasma, urine, and fecal samples. A Tri-Carb™ 3100 TR liquid scintillation counter (PerkinElmer, IL, USA) was used for the LSC measurements. Samples were re-homogenized and/or reanalyzed if the coefficient of variation of replicate radioactivity measurements was above 20%.

**Whole Blood Samples**

In a triplicate analysis, an aliquot of the whole blood sample (300 µL; weighed [determined at the clinical site]) was transferred into a 20-mL glass vial. Tissue solubilizer (1 mL of Solvable™; PerkinElmer, The Netherlands) was added, and the sample was incubated for 60 minutes at 60°C in a water bath. After cooling the vial in a cold water bath, 100 µL of 0.1 M Titriplex® (VWR, The Netherlands) was added, and the sample was decolorized by adding three times the volume of 75 µL of hydrogen peroxide (VWR, The Netherlands) in steps of 5 min. After incubation for 15 minutes at room temperature, the mixture was heated again for 15 minutes at 45°C in a water bath, followed by heating for 30 minutes at 60°C. After cooling the vial in a cold water bath, 18 mL of the scintillation cocktail (Ultima Gold™; PerkinElmer, The Netherlands) was added. After vortex mixing for at least 5 seconds, the vial was placed in an ultrasonication bath at room temperature for 5 minutes. The vial was placed in the liquid scintillation counter for at least 35 hours before counting.

**Plasma Samples**

In a triplicate analysis, an aliquot of the plasma sample (250 µL; weighed [determined at the clinical site]) was transferred into a 20-mL glass vial (PerkinElmer, The Netherlands), and 5 mL of the scintillation cocktail (Ultima Gold™; PerkinElmer, The Netherlands) was added. After vortex mixing for at least 5 seconds, the sample was placed in the liquid scintillation counter for at least 30 minutes before counting.

**Urine Samples**

In a duplicate analysis, an aliquot of the urine sample (1 mL) was transferred to a 7-mL glass vial (PerkinElmer, The Netherlands), and 5 mL of the scintillation cocktail (Ultima Gold™; PerkinElmer, The Netherlands) was added. After vortex mixing for at least 5 seconds, the sample was placed in the liquid scintillation counter for at least 30 minutes before counting.

**Fecal Samples**

Four accurately weighed aliquots of approximately 500 mg of the feces homogenate sample were dried in a stove at 50°C for at least 3 hours. After the addition of 100 µL of Combustaid™ (PerkinElmer, The Netherlands) to the dried homogenates, the samples were combusted in a sample oxidizer model 307 (PerkinElmer, The Netherlands). Subsequently, 7 mL of Carbo-Sorb E™ (PerkinElmer, The Netherlands) was used as an absorber agent for carbon dioxide. At the end of the combustion cycle, the absorber was mixed with 13 mL of the scintillation cocktail PermaFluor E™ (PerkinElmer, The Netherlands). The samples were placed in the liquid scintillation counter for at least 30 minutes before counting.

**Excretion of Radioactivity and Mass Balance**

[$^{14}$C] radioactivity data for excreta provided by the clinical site laboratory were used to calculate the percentage of dose excreted through urine or feces as well as the total percentage of dose recovered (“mass balance”). The [$^{14}$C] radioactivity data based on LSC analysis were provided by PRA Health Sciences (Bioanalytical Laboratory, Groningen, The Netherlands). The raw data

reported included all individual measurements, background data, validation data, and mass balance calculations.

## 4. Determination of Plasma Icenticaftor Concentrations

Plasma icenticaftor concentrations were quantified using a validated liquid chromatography tandem mass spectrometry (LC-MS/MS) bioanalytical method at Veeda Clinical Research Pvt. Ltd (Gujarat, India). Samples were prepared by adding 50 µL of internal standard ([$^{13}CD_3$]icenticaftor) to 100 µL of aliquots of the collected plasma samples, followed by protein precipitation with acetonitrile and centrifugation. Following this, 2-µL aliquots of the supernatant were injected into the LC-MS/MS system.

High-performance liquid chromatography (HPLC) was performed using a Shimadzu system (Shimadzu Corporation, Kyoto, Japan). The separation was performed on a Kinetex C18 column (50 × 4.6 mm, 2.6 µm particle size; Phenomenex, Torrance, CA, USA) maintained at 40°C. The analysis involved isocratic elution using Mobile Phase A (0.1% acetic acid in water) and Mobile Phase B (acetonitrile), with a flow rate of 1 mL/min. The LCMS-8050 Mass Spectrometer (Shimadzu Corporation, Kyoto, Japan) was coupled to the HPLC system through an electrospray interface, operated in the positive ionization mode. The temperature of the source interface was set at 300°C. The following transitions were monitored: for icenticaftor *m/z* 362.1 → *m/z* 191.0 (100 ms dwell time); for [$^{13}CD_3$]icenticaftor, *m/z* 366.0 → *m/z* 195.1 (100 ms dwell time). All data were acquired using LabSolutions version 5.72 B software (Shimadzu Corporation, Kyoto, Japan).

The bioanalytical method was fully validated according to current US Food and Drug Administration (FDA) and European Medicines Agency (EMA) bioanalytical guidelines as a quantitative method in terms of linearity, specificity, accuracy, precision, recovery, matrix effect, and stability. The calibration range was 1.00–2000 ng/mL, with a lower limit of quantification (LLOQ) of 1.00 ng/mL (linear regression with $1/x^2$ weighting factor). Concentrations were

expressed in mass per volume units and referred to the free base. Concentrations below the LLOQ were reported as “zero,” and missing data were labeled as such.

## 5. Determination of Metabolite Profiles in Plasma, Urine, and Feces

### Plasma Samples

For metabolite profiling in plasma, identical aliquots of plasma were combined across time points (time pools). Plasma extracts were obtained using protein precipitation with two and a half volumes of acetonitrile. Samples were frozen at −20°C for at least 24 hours and ultrasonicated for 5 minutes in an ice bath. After centrifugation (18,000 ×*g*, for 30 min, at 4°C), the supernatants (S1) were removed. The remaining pellets (P1) were solubilized with a mixture of 1 mL of Solvable (PerkinElmer)/isopropanol solution mixture (2:1; v:v) and subsequently neutralized with 0.5 mL of 2 M hydrochloric acid, after which 17.5 mL of Ultima Gold™ XR was added and LSC analysis was performed. The radioactivity in the supernatants S1 was measured in triplicate (aliquots of 100–150 µL). A single 400 µL aliquot was used for the 48-hour supernatant because of the low levels of radioactivity. For each corresponding time point, the S1 extracts were combined, and the samples were concentrated to dryness using nitrogen within an ice bath. Residues were separately reconstituted with water and acetonitrile (4:1; v:v), vortexed, and centrifuged for 30 minutes at 18,000 ×*g*. The radioactivity in the reconstituted samples was measured with 15 µL aliquots in triplicate. The remaining pellets (P2) were solubilized with a mixture of 0.5 mL of Solvable (PerkinElmer)/isopropanol (2:1; v:v) and subsequently neutralized with 0.25 mL of 2 M hydrochloric acid, after which 17.5 mL of Ultima Gold™ XR was added and LSC analysis was performed. The reconstituted plasma samples were stored at −80°C until HPLC-MS analysis with offline radiodetection (Agilent Technologies, Waldbronn, Germany). Total recovery of the plasma extractions after sample preparation ranged from 92.5% to 94.5% for all time points for up to 24 hours. For the last analyzed time point (48 hours), total recovery after sample preparation was 73.0%. Plasma extract (100–150

μL) was injected into the HPLC-MS system with offline radioactivity detection. The recovery of the radioactivity after HPLC analysis was measured for a representative sample and found to be complete (100.8%).

**Urine Samples**

Urine samples collected from each subject were pooled across 0–96 hours, and a pooled sample was prepared by combining identical volume percentages of the different urine fractions. This pool represented 97.9% of the radioactivity excreted in urine. The urine pools were stored below −80°C until HPLC-MS analysis with offline radioactivity detection. An aliquot of 150 μL from the urine pool was injected into the HPLC-MS system, with offline radioactivity detection. HPLC recovery was found to be complete (100.0%).

**Fecal samples**

Fecal samples collected from each subject were pooled across 0–120 hours, and a homogenate pool was prepared by combining identical percentages of the different homogenate fractions. This pool represented 96.8% of the radioactivity excreted in feces. The fecal pool was extracted by adding 6 mL of acetonitrile to a weighted aliquot of the fecal homogenate (~1.7 g). The mixture was incubated at 11°C under agitation at 600 rpm for 8 hours using an Eppendorf Thermomixer C and then centrifuged at 12,000 ×*g* for 20 minutes at 4°C. The supernatant S1 was removed, and the pellet was further extracted with 0.5 mL of water and sonicated over ice for 5 min, followed by addition of 2.5 mL of acetonitrile, and the contents were agitated at 600 rpm for 4 hours. The mixture was centrifuged at 12,000 ×*g* for 20 minutes at 4°C. The supernatant (S2) was separated from the pellet (P2) and added to S1 extract to generate S3. The extraction process recovered 82.8% of the radioactivity in the sample. Residual radioactivity in P1 was measured by solubilizing the pellet with a mixture of 8 mL of Solvable (PerkinElmer)/isopropanol (2:1; v:v), which was then agitated at 600 rpm (Eppendorf Thermomixer C at 60°C), and neutralized with 1 mL of 2 M hydrochloric acid. The mixture was

split into 16 × 0.5 mL aliquots and mixed with Ultima Gold™ XR, and LSC analysis was performed. A weighed aliquot of 4 mL feces extract was concentrated under nitrogen to dryness and reconstituted to 500 µL with 50% water and 30% acetonitrile and 20% dimethyl sulfoxide (DMSO). Radioactivity was measured with aliquots of 20 µL in triplicate. The reconstitution process recovered 93.7% of the extracted radioactivity, yielding a total recovery of 77.6% using the pooled feces (0–120 hours) after sample preparation. An aliquot from the pooled feces reconstituted sample (100 µL) was evaluated using HPLC-MS analysis with offline radioactivity detection. HPLC recovery of the feces reconstituted sample was measured using the same injection volume. HPLC recovery was found to be complete (95.3%).

All samples were assayed for $^{14}C$-radioactivity using the LSC counter model Tri-Carb 2200CA or Tri-Carb 3170TR/SL (Packard Instruments, Meriden, CT, USA).

**HPLC Instrumentation for Metabolite Pattern Analysis**

The Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) included a binary capillary pump, a column oven, a degasser, and an ultraviolet (UV)-visible spectroscopy diode array detector. The operating software program was Agilent ChemStation B.04.02 [118]. Samples of up to 250 µL were injected into a 350 µL sample loop using a HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland). The components were separated at 40°C on an ACE C18 analytical column (150 × 4.6 mm, 3 µm particle size; Advanced Chromatography Technologies, Aberdeen, Scotland, UK) protected by a 20 × 2.1 mm guard column of the same stationary phase.

The mobile phase consisted of ammonium carbamate 10 mM (pH 7.5) (Mobile Phase A) and acetonitrile (Mobile Phase B). The flow rate was 900 µL/min. The gradient used was as follows: 0–5 min, 5% B isocratic; 5–45 min, 55% B linear gradient; 45–55 min, 100% B linear gradient; 55–60 min, 100% B linear gradient; 60–61 min, 5% B linear gradient; and 61–65 min, 5% B isocratic. For hydrogen/deuterium exchange experiments, the water in Mobile Phase A was

replaced by deuterium oxide. For improved separation of the metabolites M8, P26.4, M9, M10, and M14, Mobile Phase A was adjusted to pH 6.5.

A time-of-flight mass spectrometer model Synapt G2-Si operated under MassLynx, version 4.1 (Waters Corporation, Manchester, UK) with electrospray in the positive ion mode and Z-spray interface with the Lock-Spray™ option was used for mass spectrometry. The reference channel of the Lock-Spray interface was operated with a solution of leucine-enkephalin (0.2 µg/mL) in acetonitrile:water:formic acid (50:50:0.1; v/v/v) at a flow rate of 5 µL/min.

## 6. Synthesis of Reference Standards

### NIR205 (M14)

Icenticaftor (125 mg, 0.346 mmol) was dissolved in dry acetonitrile:dichloromethane (1:1, 80 mL), and 1 M boron tribromide solution (prepared in dichloromethane, 10 mL) was slowly added. After stirring for 2 hours, the reaction was quenched with methanol (10 mL). Solvents were evaporated under reduced pressure to a final volume of 5 mL. The residue was mixed with water (200 mL), and the pH was adjusted to 8.5 with a 25% aqueous solution of ammonia. Compounds were extracted into ethyl ether (2 × 150 mL). Organic layers were combined and evaporated to dryness, and the residual raw product was dissolved in acetonitrile (5 mL). The raw product solution was purified using RP18 chromatography under the following conditions: Waters XBridge™ (Milford, MA, USA) C18 column (30 × 100 mm), Phase A = 0.05% aqueous acetic acid, Phase B = acetonitrile, linear gradient from 15% B to 95% B in 60 min, flow rate = 35 mL/min, detection at 252 nm. The product fraction was dried by lyophilization. The obtained product NIR205 (35 mg, 0.099 mmol, 29% yield) was characterized by LC-MS and nuclear magnetic resonance (NMR) spectroscopy: LC-UV purity >99%, electrospray ionization (ESI)–346.1 Da (**Supplemental Fig. 3**).

**IOD568 (M5)**

The synthesis of IOD568 (M5) was conducted at Hypha Discovery Ltd (Abingdon, UK) in a two-step proprietary chemical glucuronidation/deprotection procedure for O-glucuronide formation using NIR205 (800 mg, 2.3 mmol) to yield the crude product as a dark reddish semisolid (3.035 g). This residue was dissolved in DMSO (12 mL) and purified using a RP18 column under the following conditions: Waters XBridge™ (Milford, MA, USA) C18 column (30 × 100 mm), Phase A = 0.1% aqueous formic acid, Phase B = acetonitrile, linear gradient from 10% Mobile Phase B to 100% Mobile Phase B in 15 min, flow = 17 mL/min, detection at 252 nm. The product fraction was dried by lyophilization. The obtained product IOD568 (272.4 mg, 0.467 mmol, 20% yield) was characterized by LC-MS and NMR analyses: LC-UV purity >95%, ESI+ 524.2 Da (**Supplemental Fig. 3**).

**CKW231 (M8) and CKW232 (M9)**

Icenticaftor (500 mg, 1.384 mmol) was used in a glucuronidation reaction with rabbit liver S9 as previously described (Kittelmann et al., 2003). Subsequent purification was performed with RP18 chromatography on a Waters XBridge™ (Milford, MA, USA) C18 column (30 × 100 mm) Phase A = aqueous 10 mM ammonium bicarbonate solution, Phase B = acetonitrile, linear gradient from 10% B to 76% B in 12 min, flow rate = 17 mL/min, with detection at 252 nm. This resulted in two metabolites (M8 and M9) partially co-eluting at 8.6 and 10.5 min, respectively. Fractions containing mainly the N-glucuronide CKW231 were re-purified using the same chromatography conditions, resulting in the lyophilized product CKW231 (98 mg, 0.179 mmol, 43% yield), which was characterized by LC-MS and NMR: LC-UV purity >95%, ESI+ 538.2 Da. Fractions containing mainly the O-glucuronide CKW232 were re-purified using the same chromatography conditions but with 0.1% formic acid as the phase modifier instead of the ammonium bicarbonate, resulting in the lyophilized product CKW232 (98 mg, 0.179 mmol, 43% yield), which was characterized by LC-MS and NMR: LC-UV purity >95%, ESI+ 538.1 Da (**Supplemental Fig. 3**).

## 7. In Vitro Phenotyping of Cytochrome P450 (CYP) Enzymes

### Incubation of [$^{14}$C]Icenticaftor with Human Liver Microsomes (HLMs) and Recombinant Human CYP Enzymes

A pool of HLMs prepared from 150 individual donors (UltraPool™ HLM 150) was obtained (BD Biosciences, MA, USA). Stock solutions of 10 mM [$^{14}$C]icenticaftor were prepared in acetonitrile. The incubations were performed in 0.1 M potassium phosphate buffer (pH 7.4, at 37°C). Typical incubations of 900 µL were prepared as follows: 45 µL of 100 mM $MgCl_2$ (5 mM final concentration), substrate (0.5, 1, 2, 3, 4, 5, 6, 7, 8, and 10 µM), and HLMs (0.5 mg protein/mL) or recombinant human CYP enzymes (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6*1, CYP2E1, CYP2J2, CYP3A4, CYP3A5, CYP4A11, CYP4F2, CYP4F3B and CYP4F12) were added to the required volume of the buffer and pre-incubated for 3 minutes at 37°C. The reaction was started by addition of 90 µL of a fresh 10 mM solution of NADPH (1 mM final concentration). The final concentration of the organic solvent was 0.5% (v/v). For some experiments, different incubation volumes were prepared by maintaining the quantity of all solutions proportional. The samples were incubated at 37°C in a thermomixer comfort (Eppendorf 5355) under agitation at 500 rpm for 30 minutes. For incubations with recombinant human CYP2A6, CYP2C9, CYP2C18, and CYP4A11, the phosphate buffer was replaced by Tris buffer (50 mM, pH 7.5).

The enzymatic reactions were stopped, and the protein was precipitated by addition of an equal volume of acetonitrile. After 30 minutes at −80°C (or overnight at −20°C), the samples were centrifuged at 30,000 ×g for 15 min, and the supernatant was removed. Aliquots of the supernatant were evaluated using LSC analysis (20, 50, or 100 µL), and the supernatant was diluted with water to obtain a final solution containing less than 10% of the organic solvent. For samples with a low substrate concentration, the supernatant was evaporated to approximately

50% of the initial volume or to dryness under a nitrogen stream at 40°C using a Liebisch™ Evaporator (Fisher Scientific, Wohlen, Switzerland) then mixed with acetonitrile and water to achieve a final solution containing less than 10% of organic solvent. The samples were evaluated using HPLC analysis combined with radioactivity detection as shown at the end of this section.

**Concentration-Dependent Biotransformation of [$^{14}$C]Icenticaftor in HLMs**

The enzyme kinetic parameters $K_m$ and $V_{max}$ (Michaelis-Menten constant [substrate concentration producing half-maximal velocity] and maximum velocity [reaction velocity at saturating substrate concentration], respectively) were determined by incubating pooled HLMs (0.5 mg protein/mL) with 10 concentrations of [$^{14}$C]icenticaftor ranging from 0.5 to 10 µM for 30 min. Enzyme kinetics was performed using established linear conditions (time, enzyme concentration), and less than 20% of the initial substrate was consumed at the end of incubations. The overall formation rates of all the oxidative metabolites were analyzed using nonlinear regression analysis considering different kinetic models (Michaelis-Menten, Hill, isoenzyme, and substrate inhibition) as provided by the enzyme kinetics module SigmaPlot (version 12.1).

**Analysis of Enzyme Kinetics**

The enzyme kinetic parameters $V_{max}$ and $K_m$ were calculated using SigmaPlot version 12.1, Enzyme Kinetics module version 1.3 software (SPSS Science Inc., Chicago, IL, USA). Intrinsic clearance ($CL_{int}$) was calculated using the following equation: $CL_{int} = V_{max}/K_m$. Half-maximal inhibitory concentration ($IC_{50}$) values were estimated by graphical extrapolation.

**Metabolic Turnover of [$^{14}$C]Icenticaftor by Recombinant Human CYP and Flavin-Containing Monooxygenase Enzymes**

Microsomes prepared from baculovirus-infected insect cells (BTI-TN-5B1-4) expressing a single human CYP or flavin-containing monooxygenase (FMO) isoenzyme were used to assess the

involvement of specific enzymes in the biotransformation of icenticaftor. Incubation experiments with a panel of 18 recombinant CYPs (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, CYP4A11, CYP4F2, CYP4F3B, and CYP4F12; 40 pmol CYP/mL) and three recombinant FMOs (FMO-1, FMO-3, and FMO-5; 0.5 mg protein/mL) were conducted with 1 µM and 8 µM [$^{14}$C]icenticaftor.

**Inhibition of [$^{14}$C]Icenticaftor Biotransformation by Chemical Inhibitors**

The oxidative metabolism of 1 µM [$^{14}$C]icenticaftor by HLMs (0.5 mg/mL) was tested in the presence of 10 chemical inhibitors: 0.156, 0.313, 0.625, 1.25, 2.5, 5, and 10 µM furafylline (CYP1A2 inhibitor), 0.0391, 0.0781, 0.156, 0.313, 0.625, 1.25, 2.5, 5, and 10 µM ticlopidine (CYP2B6/CYP2C19 inhibitor), 0.00781, 0.0156, 0.0313, 0.0625, 0.125, 0.25, 0.5, 1, and 2 µM montelukast (CYP2C8 inhibitor), 0.0781, 0.156, 0.313, 0.625, 1.25, 2.5, and 5 µM sulfaphenazole (CYP2C9 inhibitor), 0.00391, 0.008, 0.0156, 0.0313, 0.0625, 0.125, 0.250, 0.50, 1, and 2 µM loratadine (CYP2C19 inhibitor), 0.00781, 0.0156, 0.0313, 0.0625, 0.125, 0.25, 0.5, 1, and 2 µM quinidine (CYP2D6 inhibitor), 1.56, 3.125, 6.25, 12.5, 25, 50, and 100 µM sodium diethyldithiocarbamate (DETC; CYP2E1 inhibitor), 0.00781, 0.0156, 0.0313, 0.0625, 0.125, 0.25, 0.5, and 1 µM ketoconazole (CYP3A4/5 inhibitor), 0.0391, 0.0781, 0.156, 0.313, 0.625, 1.25, 2.5 and 5 µM azamulin (CYP3A4/5 inhibitor), and 2.5, 5, 10, 20, 40, 80, and 160 µM methimazole (FMO inhibitor). After 30 minutes incubation at 37°C, the supernatant of the incubation was evaluated using HPLC analysis with radioactivity detection.

**HPLC conditions**

| | |
|---|---|
| Instrument | Agilent 1206 HPLC system with a binary pump (model G1312B, Agilent Technologies, Waldbronn, Germany) |
| Column | Aquity UPLC HSS T3; 1.8 µm, 5 × 2.1 mm (Waters, Milford, MA, USA) |

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|---|---|---|---|
| Pre-column | Aquity UPLC HSS T3; 1.8 µm, 150 × 2.1 mm (Waters, Milford, MA, USA) | | |
| Temperature | 60°C (HPLC gradient [A]); 40°C (HPLC gradient [B]) | | |
| Injection | 900 µL sample loop | | |
| Mobile phase | A: ammonium carbamate 10 mM, pH 7.5<br>B: acetonitrile | | |
| Flow rate | 0.2 mL/min (HPLC Gradient [A]); 0.1 mL/min (HPLC Gradient [B]) | | |
| HPLC Gradient A (used for time-dependent oxidative metabolism) | Gradient time (min) | % Mobile Phase A | % Mobile Phase B |
| | 0 | 80 | 20 |
| | 12 | 80 | 20 |
| | 16 | 5 | 95 |
| | 23 | 5 | 95 |
| | 30 | 80 | 20 |
| | 45 | 80 | 20 |
| HPLC Gradient B (used for other assays) | Gradient time (min) | % Mobile Phase A | % Mobile Phase B |
| | 0 | 95 | 5 |
| | 3 | 95 | 5 |
| | 67.5 | 5 | 95 |
| | 80 | 5 | 95 |
| | 85 | 95 | 5 |
| | 120 | 95 | 5 |
| UV detection | 250 nm | | |

## 8. In Vitro Phenotyping of Uridine Diphosphate Glucuronosyltransferase (UGT) Isozymes

### Incubation of [$^{14}$C]Icenticaftor With HLMs and Recombinant Human UGTs

A pool of HLMs prepared from 50 individual donors was obtained from BD Biosciences (MA, USA). Stock solutions of 50 mM [$^{14}$C]icenticaftor were prepared in acetonitrile. HLMs (stock solution 20 mg protein/mL) or recombinant UGTs were pre-incubated with 50- or 200-µg alamethicin/mg protein as follows: the required volume of protein solution was mixed with an appropriate volume of alamethicin stock solution (50 mg/mL in methanol) and incubated for 5 minutes at room temperature. After the solution mixture was diluted with 250 mM ice-cold sucrose solution in water (for HLMs) or 50 mM Tris buffer (pH 7.5 for recombinant UGTs), the diluted solution was kept on ice for 20–30 minutes until further use. The methanol content in the pre-incubated protein solution was 1%.

The incubations were conducted in 50 nM Tris buffer (pH 7.5, at 37°C). Typical incubations of 400 µL were prepared as follows: 20 µL of 100 mM $MgCl_2$ (5 mM final concentration), substrate (1 µM and 10 µM), and pre-incubated proteins (15 µL of alamethicin stock solution mixed with 750 µL HLM stock) were added to an appropriate volume of the buffer and pre-incubated for 3 minutes at 37°C in a thermomixer comfort (Eppendorf 5355). The reaction was started by addition of uridine 5′-diphosphoglucuronic acid (UDPGA) in 50 mM Tris buffer, pH 7.5 (5 mM final concentration) and incubated for 0, 10, 20, 30, 45, 60, 90, 120 and 180 min at 37°C and 500 rpm or for HLM protein-dependency with final HLM concentrations of 0, 0.2, 0.3, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0 and 2.5 mg/mL. For some experiments, different incubation volumes were prepared by maintaining the quantity of all solutions proportional.

The enzymatic reactions were stopped and the protein was precipitated by addition of an equal volume of acetonitrile. After 30 minutes at −80°C (or overnight at −20°C), the samples were

centrifuged at 30,000 ×*g* for 15 min, and the supernatant was collected. Aliquots (20 or 50 µL) were evaluated using LSC analysis (Tri-Carb 2500TR, Packard Instruments, Meriden, CT, USA) after mixing with 10 mL of the LSC cocktail. The remaining supernatants were evaporated to dryness or ~250 µL was dried under a nitrogen stream at 40°C using a Liebisch Evaporator (Fisher Scientific, Wohlen, Switzerland), after which acetonitrile and water were added to achieve a final solution containing less than 20% of the organic solvent. Samples were evaluated using HPLC analysis combined with radioactivity detection.

**Concentration-Dependent Biotransformation of [$^{14}$C]Icenticaftor in HLMs**

The enzyme kinetic parameters $K_m$ and $V_{max}$ were determined by incubating pooled HLMs (0.5 protein/mL) with 1, 2, 3, 5, 7, 10, 15, 20, 50, 80, and 100 µM of [$^{14}$C]icenticaftor for 15–20 min (500 rpm, 37°C). Enzyme kinetics was performed using the established linear conditions (time and enzyme concentration), and less than 20% of the initial substrate was consumed at the end of incubations. The overall formation rates of all glucuronide conjugates were analyzed using nonlinear regression analysis considering different kinetic models (Michaelis-Menten, Hill, isoenzyme, and substrate inhibition) as provided by the Enzyme Kinetics module, SigmaPlot.

**Analysis of Enzyme Kinetics**

The enzyme kinetic parameters $K_m$ and $V_{max}$ were calculated using SigmaPlot version 8.0, Enzyme Kinetics module version 1.1 software (SPSS Science Inc., Chicago, IL, USA). $CL_{int}$ was calculated using the following equation: $CL_{int} = V_{max}/K_m$.

**Determination of Unbound Fraction ($f_{u,mic}$) of Icenticaftor in HLMs**

[$^{14}$C]Icenticaftor (1 and 20 µM) was mixed with pooled HLMs (0.0039, 0.0078, 0.0156, 0.0313, 0.0625, 0.125, 0.25, 0.5, or 1 mg protein/mL), phosphate buffer (50 mM), and magnesium chloride (5 mM). Samples were incubated for 10 min at 37°C in a water bath under agitation at 150 rpm. After vortexing, 50-µL aliquots of each sample were removed for LSC analysis. The rest of the sample (950 µL) was centrifuged at 200,000 ×*g* for 3.5 hours at 37°C. Aliquots of 800

μL were removed carefully from the supernatants of the samples, and 50 μL of the supernatant aliquot was used for LSC analysis. LSC samples (50 μL) were mixed with 10-mL Irga-Safe Plus (Perkin-Elmer, Boston, MA, USA) and measured with the LSC (Tri-Carb 2500TR; Perkin-Elmer, Boston, MA, USA).

The unbound fraction of icenticaftor in microsomal incubations ($f_{u,mic}$) was determined by ultracentrifugation at icenticaftor concentrations of 1 and 20 μM. Experimental values for $f_{u,mic}$ decreased from 0.814 ± 0.165 to 0.477 ± 0.097, with increasing protein concentrations ranging from 0.0039 mg/mL to 1 mg/mL and could be represented by a fitting function:

$f_{u,mic}$ = 0.2571 × [mg of microsomal protein]$^2$ − 0.5798 × (mg of microsomal protein) + 0.7995, with $R^2$=0.9812 and “protein” representing the microsomal protein concentration

Accordingly, the determined apparent $K_m$ values were corrected for microsomal binding based on the protein concentration used in the incubation.

**Biotransformation of [$^{14}$C]Icenticaftor by Recombinant Human UGT Isozymes**

Microsomes prepared from baculovirus-infected insect cells (BTI-TN-5B1-4) expressing a single human UGT isozyme (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17) were used to assess the involvement of specific isoenzyme in [$^{14}$C]icenticaftor glucuronidation at 4- and 25-μM concentrations.

**HPLC Conditions**

| | |
|---|---|
| Instrument | Agilent 1100 HPLC system with a binary pump (model G1312A, Agilent Technologies, Waldbronn, Germany) |
| Column | Aquity UPLC HSS T3; 1.8 μm, 150 × 2.1 mm (Waters, Milford, MA, USA) |

| | | | |
|---|---|---|---|
| Pre-column | Aquity UPLC HSS T3; 1.8 µm, 5 × 2.1 mm (Waters, Milford, MA, USA) | | |
| Temperature | 60°C | | |
| Injection | 900 µL sample loop | | |
| Mobile phase | A: ammonium carbamate 10 mM, pH 7.5<br>B: acetonitrile | | |
| Flow rate | 0.2 mL/min | | |
| HPLC Gradient A (used for time-dependent oxidative metabolism) | Gradient time (min) | % Mobile Phase A | % Mobile Phase B |
| | 0 | 80 | 20 |
| | 12 | 80 | 20 |
| | 16 | 5 | 95 |
| | 23 | 5 | 95 |
| | 24 | 80 | 20 |
| | 40 | 80 | 20 |
| UV detection | 250 nm | | |

**9. In Vitro Biotransformation in Mouse, Rat, Monkey, and Human Hepatocytes**

Metabolic pathways across species were elucidated by incubating mouse, rat, monkey, and human hepatocytes with 1, 10, and 30 µM concentrations of [$^{14}$C]icenticaftor. A 1.0 mM stock solution of [$^{14}$C]icenticaftor in DMSO was used for the 1 µM incubation and a 13.7 mM stock solution of [$^{14}$C]icenticaftor in DMSO was used for the 10 µM and 30 µM incubations. Cryopreserved pooled hepatocytes (male mouse, ICR/CD-1; male rat, Sprague Dawley; male monkey, cynomolgus; humans of both genders) were obtained from Bioreclamation In Vitro

Technologies (Baltimore, MD, USA). Thawed and centrifuged hepatocytes were suspended in In Vitro GRO Krebs-Henseleit buffer, and the cell density was adjusted to ~l × l$0^6$ viable cells/mL after viability determination. The incubations were started by addition of [$^{14}$C]icenticaftor as a 13.7 mM solution in DMSO (2.2 µL/mL hepatocytes) for the 30 µM incubations, (0.7 µL/mL hepatocytes) for the 10 µM incubation and as a 1.0 mM solution in DMSO (1.0 µL/mL hepatocytes) for the 1 µM incubation. The initial concentrations of [$^{14}$C]icenticaftor in the incubations were 30 µM (64.4 kBq/mL), 10 µM (21.5 kBq/mL), and 1 µM (2.15 kBq/mL). The final concentration of DMSO in all incubations was below 0.5% (v/v). Incubations were performed at 37°C for 0, 4, and 24 hours, stopped with two volumes of acetonitrile, and stored at −20°C until assessment of radioactivity. Before radioactivity measurement, samples were thawed and centrifuged for 15 minutes at 10,000 ×*g*, followed by removal of the supernatant (S1). The pellet (P1) was washed with 300 µL of water:acetonitrile (1:2 v/v) solution mixture by vortexing and a short sonication step. The process was repeated, and the resultant supernatant (S2) was combined with S1 to generate S3. Radioactivity was measured in S3 using two aliquots of 50 µL and 15 mL IrgaSafe plus scintillation cocktail (PerkinElmer, Waltham, MA, USA). The pellet (P2) was dissolved in 500 µL of solvable:isopropanol 2:1 (v/v) and neutralized with 500 µL of 2 M HCl. The tube was inserted into a 20 mL scintillation vial and 15 mL IrgaSafe plus scintillation cocktail was added. A 500 µL aliquot of S3 was transferred to a HPLC vial, and the contents were evaporated to ~10 µL under nitrogen. Subsequently, 100, 500, or 1000 µL water:acetonitrile 9:1 (v/v) was added to the 1, 10, or 30 µM samples, respectively.

The samples were evaluated using HPLC analysis with offline radioactivity detection. The HPLC system used was Agilent model 1200 (Agilent Technologies, Waldbronn, Germany) equipped with a binary capillary pump (model G1376A), a diode array detector (model G1315D), and a degasser (model G1379B). The operating software program for the HPLC system was ChemStation (version 04.02; Agilent Technologies). Volumes up to 100 µL were injected into a 350 µL sample loop using a HTS PAL autosampler (CTC, Zwingen, Switzerland). The

components were separated at 40°C on an ACE C18 analytical column (150 × 4.6 mm, 3 µm particle size; Advanced Chromatography Technologies, Aberdeen, Scotland, UK) protected using a 20 × 2.1 mm guard column of the same stationary phase. The mobile phase consisted of ammonium carbamate 10 mM (pH 7.5) (A) and acetonitrile (B). The flow rate was 900 µL/min. The gradient used was: 0–5 min, 5% B isocratic; 5–45 min, 55% B linear gradient; 45–55 min, 100% B linear gradient; 55–60 min, 100% B linear gradient; 60–61 min, 5% B linear gradient; and 61–65 min, 5% B isocratic. For hydrogen/deuterium exchange experiments, the water in mobile phase A was replaced by deuterium oxide.

A time-of-flight mass spectrometer model Synapt G2-Si operated under MassLynx, version 4.1 (Waters Corporation, Manchester, UK) with electrospray in the positive ion mode and Z-spray interface with the Lock-Spray™ option was used for mass spectrometry. The reference channel of the Lock-Spray interface was operated with a solution of leucine-enkephalin (0.2 µg/mL) in acetonitrile:water:formic acid (50:50:0.1; v/v/v) at a flow rate of 5 µL/min. For offline radioactivity detection, the effluent was collected in 9.6 s/fraction in four 96-well LumaPlates, the fractions were evaporated to dryness and the radioactivity counts were determined in a microplate scintillation counter model TopCount NXT (Packard Instruments, Meriden, CT, USA). Metabolite structures were characterized using LC-MS or LC-MS/MS and deuterium exchange. Samples were assayed for $^{14}$C-radioactivity in a LSC counter model Tri-Carb 2200CA or Tri-Carb 3170TR/SL (Packard Instruments, Meriden, CT, USA).

**10. Rat ADME Study**

The ADME of icenticaftor in rats was assessed after a single oral/by mouth or intravenous (IV) dose of [$^{14}$C]icenticaftor. Relevant details for metabolic profiling and structural characterization of the two groups of rats are described below.

**Animals**

| | |
|---|---|
| Animal license numbers | No. 18 and No. 20, Kantonales Veterinäramt Basel |
| Species, strain, sex | Wistar rats (Han:WIST), Albino, males<br>Long Evans rats (crt:(LE)BR), pigmented, males |
| Supplier | Han:WIST: Harlan, The Netherlands<br>crt:(LE)BR: Charles River Laboratories, Germany |
| Water/feeding | Free access to tap water and NAFAG pellets No. 890 (Eberle NAFAG AG, Gossau, Switzerland) throughout the study |
| Environmental conditions | 22 ± 2°C |

**Animal treatment groups and detailed information**

| Treatment group | Route/dose | Number of animals per group | Rat strain (all male) | Weight (g)/age (weeks) | Housing after dosing |
|---|---|---|---|---|---|
| Metabolism/excretion | Oral/10 mg/kg | 3 | Han:WIST | 261–280/9–10 | Metabolism cages |
| Bile excretion | IV/3 mg/kg | 3[a] | Han:WIST | 261–287/9–10 | Metabolism cages |

IV, intravenous.

[a]Four animals cannulated, and three animals were dosed.

**Bile Duct Cannulation**

Rats were anesthetized with an oxygen/isoflurane mixture (97/3, v/v; Forene®, Abbott AG, Baar, Switzerland). For pre- and post-surgery analgesia, meloxicam was administered subcutaneously 1 hour before surgery and during the recovery phase (1–2 mg/kg every 24 hours up to 72 hours after bile duct cannulation). The compound was administered 72 hours after bile duct cannulation.

**Radiolabeled Study Drug Formulations and Dosing**

[$^{14}$C]Icenticaftor was supplied by the Isotope Laboratory of Novartis Pharma AG, Basel, Switzerland (radiochemical purity >99.5%). The chemical structure and position of the radiolabel are shown in **Fig. 1**.

For oral administration, an aqueous suspension of 0.5% (w/v) methyl cellulose containing 0.5% (v/v) Tween 80/ethanol 36:1 (w/w) was used. The solid compound was dispersed in ethanol by stirring and sonication, followed by the addition of the methyl cellulose/Tween 80 solution. During the dosing procedure, the suspension was stirred continuously. The vehicle used for intravenous dosing was 5.0% (w/v) aqueous glucose containing 1% (v/v) Tween 80/ethanol 9:1 (w/w). The solid compound was dissolved in ethanol by stirring and sonication, and the glucose/Tween 80 mixture was added.

The dosing solutions were prepared as listed below.

| Treatment group | [$^{14}$C]Icenticaftor (mg) | Ethanol (g) | Vehicle (g) | Concentration (mg/g) | RA in DS (MBq/g) | Specific RA (MBq/mg) |
|---|---|---|---|---|---|---|
| Metabolism/ excretion (oral) | 23.5 | 0.317 | 11.5[a] | 1.99 | 3.84 | 1.93 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Bile excretion (IV) | 4.62 | 0.329 | 2.71[b] | 1.52 | 6.69 | 4.41 |

DS, dosing solution/suspension; IV, intravenous; RA, radioactivity.

[a]0.5% (w/v) methyl cellulose containing 0.5% (v/v) Tween 80.

[b]5.0% (w/v) aqueous glucose containing 1% (v/v) Tween 80.

The dosing suspension for oral administration was administered by gavage to conscious rats. The nominal dose was 10 mg/kg. For intravenous administration, the dosing solution was administered as a bolus into the vena femoralis of rats anesthetized by inhalation of an oxygen/isoflurane (Forene) mixture (97/3, v/v). The nominal dose was 3 mg/kg.

**Sample Collection**

For rats in the metabolism/excretion group (oral dosing), blood samples (500 µL) were collected sublingually at 3, 8, 24, 48, 96, and 168 hours post dosing from rats that were anesthetized by inhalation of an oxygen/isoflurane (Forene) mixture (97/3, v/v). The collected blood was centrifuged within 10 minutes after collection (3000 ×*g*, for 10 min, room temperature). Plasma was separated, and an aliquot of 20 µL was removed, weighed, and assayed for radioactivity by LSC analysis. The remaining plasma was stored at approximately −80°C until analysis by Biotransformation (Basel, Switzerland).

Urine samples were collected on dry ice daily up to 96 hours then at 96–168 hours post dose. After each urine collection, the collection vial was rinsed with 3–5 mL of water and this water was added to the respective urine sample. All portions collected (Days 5 to 12, 0–168 h) were cooled to below 10°C (up to 30 minutes in an ice bath). The pH was determined using pH indicator strips and if the result was below the range pH 6.5 to pH 7.0, then 5 M sodium hydroxide was added to increase to pH 7.0. Two aliquots of each urine sample (~0.05 g) were

removed, weighed, and processed for determination of radioactivity. Remaining urine samples were stored at −80°C until analysis by Biotransformation (Basel, Switzerland). Feces samples were collected at room temperature daily for up to 96 hours, then 96–168 hours post dose. Each fecal sample was weighed and a 1% aqueous carboxymethyl cellulose solution was added (the volume being approximately 10 times the weight of the fecal sample). The mixture was homogenized thoroughly, and three aliquots of each homogenate (each 2–3 g) were removed, weighed, and processed for the determination of radioactivity. The remaining fecal homogenate samples were stored at −20°C until analysis by Biotransformation (Basel, Switzerland).
For biliary excretion analysis in rats that received intravenous dosing, bile samples were collected on ice at 0–6, 6–24, and 24–48 hours post dose. Two aliquots of each bile sample (~0.05 g) were removed, weighed, and processed for the determination of radioactivity. The remaining bile samples were stored at −80°C until analysis by Biotransformation (Basel, Switzerland). Urine and fecal samples were collected according to the method described above.

**Determination of Radioactivity**

In LSC analysis, radioactivity was measured in aliquots of biological samples using 2500 TR liquid scintillation counters (Packard Instruments, Meriden, CT, USA).

**Sample Preparation for Metabolite Profiling**

***Plasma***

For each time point, aliquots of 100 mg of plasma were combined, and the final pool was weighed. Acetonitrile was added to the plasma pool at a ratio of 2:1 (v/v). The suspension was vortex mixed and stored at 4°C for at least 4 hours. The mixture was centrifuged at 36,670 ×*g* for 20 minutes (3K30 Centrifuge; SIGMA, Osterode am Harz, Germany), and the supernatant decanted and evaporated under a nitrogen stream to a third of the volume. The concentrated

extract was centrifuged at 36,670 ×*g* for 20 min and the supernatant decanted. Radioactivity was measured in duplicate using LSC analysis.

The pellet was resuspended in one volume of water by sonication and re-extracted as described above. Radioactivity was measured in the supernatant using LSC analysis in duplicate. The concentrated extracts were combined and 5% acetonitrile (in volume) was added. Radioactivity was measured in the remaining pellet using the solubilizing method with soluene/isopropanol using LSC analysis to determine extraction recoveries.

***Urine***

Pools of 0–96 hour samples (metabolism/excretion group) and of 0–48 hour samples (bile excretion group) were prepared from combined urine aliquots. The final weight of each pool was recorded, and radioactivity was measured using LSC analysis in triplicate. An aliquot of the urine pool was centrifuged at 36,670 ×*g* for 20 minutes (3K30 Centrifuge; SIGMA, Osterode am Harz, Germany). The supernatant was decanted, and 5% acetonitrile was added, and radioactivity was measured using in triplicate by LSC analysis.

***Feces***

Pools from 0–96 hour samples (metabolism/excretion group) and 0–48 hour samples (bile excretion group) were prepared from combined fecal aliquots. The final weight of each pool was recorded, and radioactivity was determined in triplicate by LSC using the solubilizing method with soluene/isopropanol. Acetonitrile was added to an aliquot of 1 mL of the fecal pool at a ratio of 4:1 (v/v), and the sample was mixed for at least 4 hours under agitation at 200 rpm. The suspension was centrifuged at 8331 ×*g* for 20 minutes (GS-15R Centrifuge, Beckman, CA, USA). The supernatant was decanted and weighed, and radioactivity was measured in triplicate using the LSC analysis. The pellet was resuspended in one volume of water by sonication, vortex mixed, and re-extracted twice as described above. Radioactivity was measured in each supernatant using LSC analysis in triplicate. The three supernatants were combined, evaporated to dryness under nitrogen flow, and reconstituted in a mixture of water/acetonitrile

(1:4, v/v). The concentrated extract was vortex mixed and sonicated in a cold ultrasound bath for 15 minutes. The mixture was centrifuged at 36,670 ×*g* for 20 minutes (3K30 Centrifuge; SIGMA, Osterode am Harz, Germany), and the supernatant was decanted and weighed. Radioactivity was measured in triplicate using LSC analysis. Radioactivity was measured in the remaining pellet using the solubilizing method with soluene/isopropanol by LSC to determine extraction recoveries.

***Bile***

A bile pool was prepared using the bile aliquots (weights of aliquots were proportional to the total weight of bile excreted during sampling time) from the 0–48 hour samples of rats in the bile extraction group. The final weight of the pool was recorded, and radioactivity was measured in triplicate using LSC analysis. An aliquot of the bile pool was centrifuged at 36,670 ×*g* for 20 minutes (3K30 Centrifuge; SIGMA, Osterode am Harz, Germany). The supernatant was decanted, and 10% acetonitrile was added. The final weight was recorded, and radioactivity was measured in triplicate using LSC analysis.

**Radioactivity Detection for Determination of Recoveries**

All radioactivity measurements were performed (for up to 10 minutes measuring time) using the LSC analyzer (Tri-Carb; Packard Instruments, Meriden, CT, USA). Radioactivity was measured in all extracted samples and pools (except for fecal pools) using weighed aliquots (up to 100 mg) and 5 mL of the scintillation cocktail (Rialuma™, Lumac, The Netherlands) in 6 mL LSC vials. All pellets and fecal pools (except for lung pellets) were solubilized with soluene/isopropanol 1:1 (v/v; up to 5 mL) and stored at 60°C overnight. The suspensions were split into smaller aliquots when needed. Hydrochloric acid (2 M; up to 0.5 mL) was added before radioactivity measurements with 18 mL of the scintillation cocktail (Rialuma™, Lumac, The Netherlands) in a 20 mL LSC vial.

**Metabolite Profiling**

Metabolite profiling in plasma, urine, and feces was performed using ultra-performance liquid chromatography (UPLC) and MS under the conditions provided below.

| **UPLC** | | |
|---|---|---|
| Instrument | Waters Acquity UPLC (Milford, MA, USA) equipped with two binary pumps, degasser, column manager, autosampler, and diode array detector (DAD) | |
| Guard column | Waters HSS T3 (Milford, MA, USA) 1.8 µm, 5 × 2.1 mm | |
| Analytical column | Waters HSS T3 (Milford, MA, USA) 1.8 µm, 150 × 2.1 mm | |
| Temperature of guard and analytical column | 40°C | |
| Injection volume | 10–50 µL injected through a 100 µL sample loop | |
| Mobile phase | A: Ammonium carbamate 10 mM, pH 7.5<br>B: Acetonitrile<br>For deuterium exchange experiments, ammonium carbamate (10 mM, pH 7.5) in deuterium oxide was used | |
| Flow rate | 500 µL/minutes | |
| Gradient | Gradient time (minutes) | % Mobile phase B |
| | 0.0–0.6 | 5 |
| | 0.6–12.5 | 5–95 |
| | 12.5–14.6 | 95 |
| | 14.6–15.2 | 95–5 |
| | 15.2–20.0 | 5 |
| UV detection | 253 and 280 nm | |

| | |
|---|---|
| Online radioactivity detection | After DAD, the effluent was split 95:5, with 95% used for online radioactivity detection and 5% used for MS analysis. Before entering the radiomonitor LB513 (Berthold Technologies GmbH & Co., KG, Bad Wildbad, Germany) equipped with a 50 µL flow cell; the effluent was mixed with 2.5 mL/minute of the liquid scintillation cocktail (Rialuma™, Lumac, The Netherlands). |
| Offline radioactivity detection | The column effluent was collected in 0.05 minute fractions on yttrium silicate scintillator–coated 384-Deepwell LumaPlates (LumaPlates; Packard BioScience, Groningen, The Netherlands), using a GX-271 Liquid Handler from Gilson (resulting in a total fractionation time of 19.2 minutes). Solvents were evaporated at room temperature, and the dry plates were processed in a microplate scintillation counter (TopCount NXT; Packard Instruments, Meriden, CT, USA.). Counting times were 1–20 minutes for each plate. The counts monitored during the three counting periods were averaged unless one of the three measurements was an outlier in the positive direction (possibly due to an electrostatic discharge), in which case only the counts from the remaining two counting periods were averaged. Moreover, a correction was made for the different background levels of the 12 photomultipliers of the microplate scintillation counter. The resulting data were converted into chromatograms and integrated using the “Radiostar” software program (Version |

| | |
|---|---|
| | V4.6, Berthold Technologies GmbH & Co., KG, Bad Wildbad, Germany). |
| **MS** | |
| Instrument | Synapt quadrupole-time-of-flight tandem MS operated under MassLynx, version 4.1 SCN639 (Waters Corporation, Manchester, UK) |
| Ionization mode | Electrospray in the positive ion mode |
| Ion source conditions | Spray capillary: 3.0 kV<br>Cone voltage: 30 V<br>Nebulizer gas: nitrogen (7 bar)<br>Cone gas: nitrogen (25 L/h)<br>Desolvation gas: nitrogen (800 L/h)<br>Source block temperature: 120°C<br>Desolvation temperature: 200°C |
| Collisional activation | Gas: argon, ($7.6 \times 10^{-3}$ mbar) (LC MS and LC MS/MS); Trap cell: 4 eV (LC MS); 15–45 eV (LC MS/MS); Transfer cell: 4 eV (LC MS and LC MS/MS) |
| Mass analysis | Mass resolution ~9000 (full width at half-maximum definition), V-mode |

## 11. References

Kittelmann M, Rheinegger U, Espigat A, Oberer L, Aichholz R, Francotte E, and Ghisalba O (2003) Preparative enzymatic synthesis of the acylglucuronide of mycophenolic acid. *Adv Synth Catal* **345:**825-829.

## 12. Supplemental Tables

**Supplemental Table 1.** Concentration of icenticaftor and its metabolites in human plasma following the administration of a single oral dose of 400 mg [$^{14}$C]icenticaftor at steady state.

| | Sample collection time (h) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 3 | 4 | 8 | 12 | 24 | 48 | | |
| Component | Concentration | | | | | | | $AUC_{0–48h}$[a] | |
| (in order of elution) | nM | | | | | | | nM*h | %[b] |
| Front | – | – | – | 6.51 | 13.2 | 14.1 | 12.3 | 534 | 0.446 |
| P2.1 | 11.5 | 15.6 | 24.4 | 24.5 | 37.2 | 34.0 | 12.9 | 1260 | 1.06 |
| M24 | – | – | 41.4 | 29.9 | 31.5 | 13.9 | 7.34 | 813 | 0.680 |
| M22 | – | – | – | 28.0 | 37.7 | 20.7 | 2.05 | 811 | 0.679 |
| **M5** | **64.7** | **469** | **610** | **537** | **443** | **230** | **56.7** | **12800** | **10.7** |
| M16 | – | – | 36.1 | 28.3 | 29.0 | 29.2 | 9.28 | 1070 | 0.897 |
| M6 | 8.71 | 11.3 | 30.8 | 11.1 | 18.0 | – | 0.624 | 303 | 0.253 |
| M7 | 30.2 | 102 | 121 | 50.9 | 38.6 | 11.4 | 2.38 | 1250 | 1.04 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **M8[c]** | **1150** | **3550** | **3950** | **1560** | **1110** | **487** | **117** | **42200** | **35.3** |
| P26.4[c] | 6.85 | 34.7 | 62.6 | 46.7 | 30.4 | 23.4 | 8.40 | 1170 | 0.980 |
| **M9[d]** | **469** | **1640** | **2130** | **662** | **376** | **150** | **42.3** | **17300** | **14.5** |
| M10[d] | 9.65 | 55.5 | 83.8 | 27.2 | 87.3 | 49.2 | 25.9 | 2310 | 1.93 |
| M14[d] | 72.5 | 165 | 157 | 26.0 | 35.2 | 30.9 | 8.55 | 1790 | 1.50 |
| M17 | 20.8 | 92.8 | 136 | 142 | 142 | 69.7 | 13.8 | 3630 | 3.04 |
| P38.5 | 18.7 | 86.7 | 79.6 | 40.6 | 7.32 | 6.32 | 1.87 | 714 | 0.598 |
| **Icenticaftor** | **1240** | **2450** | **3150** | **610** | **360** | **162** | **38.3** | **22100** | **18.5** |
| Sum of additional components[e] | 0.311 | 0.00 | 0.00 | 0.383 | 25.1 | 12.4 | 7.45 | 515 | 0.430 |
| Total detected | 3110 | 8670 | 1060 | 3830 | 2820 | 1340 | 367 | 111000 | 92.6 |
| Lost during sample processing | 206 | 534 | 860 | 223 | 177 | 93.5 | 136 | 8870 | 7.4 |
| Lost during HPLC | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total radiolabeled components in original sample | 3320 | 9200 | 1150 | 4050 | 2990 | 1440 | 502 | 120000 | 100 |

–, not detected; $AUC_{0-48h,}$ area under the concentration-time curve from time zero to 48 hours; HPLC, high-performance liquid chromatography.

Bold text denotes major components.

[a]Calculated using the linear trapezoidal method; concentrations at time zero taken as zero.

[b]Percentage of total radiolabeled components in the original sample.

[c]Percent radioactivity of components derived from percent radioactivity of resolved chromatography for M8 and P26.4 (10 mM ammonium carbamate at pH 6.5). This was applied to all plasma sample timepoints.

[d]Percent radioactivity of components derived from percent radioactivity of resolved chromatography for M9, M10, and M14 (10 mM ammonium carbamate at pH 6.5). This was applied to all plasma sample timepoints.

[e]Sum of the residual radioactive peaks. (All individual peaks have $AUC_{0-48h}$ ≤0.14%.)

**Supplemental Table 2.** Percentage of icenticaftor and its metabolites in human urine and feces.

| | Excretion (% of dose) | | |
|---|---|---|---|
| | Urine | Feces | Total |
| Component | 0–96 hours | 0–120 hours | |
| Front | – | 0.085 | 0.085 |
| P3.6 | 0.252 | – | 0.252 |
| M1 | 1.15 | – | 1.15 |
| M27 | 0.729 | – | 0.729 |
| M3 | 0.216 | – | 0.216 |
| M22 | 1.04 | – | 1.04 |
| **M5** | **11.0** | **–** | **11.0** |
| M28 | 0.711 | – | 0.711 |
| M29 | 0.306 | – | 0.306 |
| M6 | 0.216 | – | 0.216 |
| M7 | 0.999 | – | 0.999 |
| **M8** | **50.1** | **–** | **50.1** |
| **M9** | **18.4** | **–** | **18.4** |
| M31 | 0.144 | – | 0.144 |
| M14 | – | 0.174 | 0.174 |
| M32 | – | 0.148 | 0.148 |
| M17 | 0.171 | – | 0.171 |
| **Icenticaftor** | **3.35** | **2.68** | **6.03** |
| M33 | – | 0.077 | 0.077 |
| Sum of additional components | 1.24[a] | 0.583[b] | 1.83 |

| | | | |
|---|---|---|---|
| Total detected | 90.0 | 3.75 | 93.8 |
| Lost during sample processing | 0.36 | 1.14 | 1.50 |
| Lost during HPLC | 0.00 | 0.185 | 0.185 |
| Total analyzed (pool 0–96 hours) | 90.40 | 5.07 | 95.5 |
| Total excretion (time period 0–216 hours) | 92.3 | 5.25 | 97.55 |

–, not detected; HPLC, high-performance liquid chromatography.

Bold text denotes major components.

[a]Sum of residual radioactive peaks. No single component had a contribution of ≥0.23% of the dose.

[b]Sum of residual radioactive peaks. No single component had a contribution of >0.10% of the dose.

**Supplemental Table 3.** Mass spectral biotransformation data (human).

A. Assignment of metabolites to peaks in radiochromatograms (listed in order of elution).

| Component | Plasma | Urine | Feces |
|---|---|---|---|
| M1 | – | MS/MS | – |
| M27 | – | MS/MS | – |
| M3 | – | MS/MS | – |
| M24 | MS/MS | MS/MS[a] | – |
| M22 | MS | MS/MS | – |
| M5 | MS/MS | MS/MS | – |
| M28 | – | MS/MS | – |
| M16 | MS/MS | MS[a] | – |
| M29 | – | MS/MS | – |
| M6 | MS/MS | MS/MS | – |
| M7 | MS/MS | MS/MS | MS |
| M8 | MS/MS | MS/MS | – |
| M9 | MS/MS | MS/MS | – |
| M14 | MS/MS | – | MS |
| M31 | MS[a] | MS/MS | – |
| M10 | MS/MS | – | – |
| M32 | – | – | MS/MS |
| M17 | MS/MS | MS/MS | – |
| Icenticaftor | MS/MS | MS/MS | MS/MS |
| M33 | – | – | MS/MS |

–, not detected; MS, mass spectrometry; MS/MS, tandem mass spectrometry.

[a]Trace level.

B. Data from LC-MS or LC-MS/MS runs of plasma, urine, and fecal samples (listed in order of elution).

| | Observed ions in LC-MS or LC-MS/MS runs (*m/z*) | | | | |
|---|---|---|---|---|---|
| | | Fragments | | | |
| Component | [M+H]+ | A* | A-CO* | B* | Additional major signals |
| M1 | 398 | | | 122 | 222[a], 205, 177, 149 |
| M27 | 428 | 235[a] | 191[a,b] | 122[a,b] | 411 $[M+H - NH_3]^+$, 247, 219[a,b], 171, 161, 129, 107 |
| M3 | 714 | | | | 696 $[M+H - H_2O]^+$, 538[a], 520, 502, 404, 362, 344 |
| M24 | 237 | 219 | 191 | 122 | 171, 107 |
| M22 | 412 | 219[a] | 191[a] | 122[a] | 394 $[M+H - H_2O]^+$, 376, 358, 314, 297, 278, 236[a], 233, 141, 95 |
| M5 | 524 | 205[a] | 177[a] | | 348[a], 330 $[M+H - H_2O - C_6H_8O^6]^+$, 122 |
| M28 | 428 | | | | 236, 234 $[M+H - H_2O - C_6H_8O_6]^+$, 219[a,b], 191[a,b], 122[a,b], 206, 148 |
| M16 | 354 | | | | 336 $[M+H - H_2O]^+$, 318, 218, 193, 165, 97 |
| M29 | 554 | | | | 536 $[M+H - H_2O]^+$, 360 $[M+H - H_2O - C_6H_8O_6]^+$, 297, 261, 233, 219[a,b], 191[a,b], 122[a,b] |
| M6 | 554 | | | | 536 $[M+H - H_2O]^+$, 518, 500, 456, 438, 420, 402, 378[a], 360 $[M+H - H_2O - C_6H_8O_6]^+$, 297, 269, 261, 233, 219[a,b], 191[a,b], 205, 141, 122[a] |
| M7 | 554 | 219[a,b] | 191[a,b] | | 362 $[M+H - O - C_6H_8O_6]^+$, 344, 208, 129 |

| | | | | | |
|---|---|---|---|---|---|
| M8 | 538 | 219[a] | 191[a] | 122[a] | 520[M+H – $H_2O$]$^+$, 502, 484, 440, 404, 362[a], 344 [M+H – $H_2O$ – $C_6H_8O_6$]$^+$, 297 |
| M9 | 538 | 219[a] | 191[a] | 122[a] | 520 [M+H – $H_2O$]$^+$, 502, 484, 440, 362[a], 344 [M+H– $H_2O$ – $C_6H_8O_6$]$^+$ |
| M14 | 348 | 205 | 177 | | 330 [M+H – $H_2O$]$^+$, 310 [M+H – $H_2O$ – HF]$^+$, 149, 122 |
| M31 | 554 | | | | 536 [M+H – $H_2O$]$^+$, 378[a], 360 [M+H – $H_2O$ – $C_6H_8O_6$]$^+$, 342, 237, 219[a,b], 191[a,b], 142, 122[a,b] |
| M10 | 378 | 235 | 207 | | 360 [M+H – $H_2O$]$^+$, 340, 215, 187, 179, 159, 147 |
| M32 | 442 | 219[c] | 191[c] | 122[c] | 362 [M+H – $SO_3$]$^+$, 344 [M+H – $SO_3$ – $H_2O$]$^+$ |
| M17 | 236 | 219 | 191 | 122 | 107 |
| Icenticaftor | 362 | 219 | 191 | 122 | 344 [M+H – $H_2O$]$^+$, 107 |
| M33 | 404 | 219[d] | 191[d] | 122[d] | 386 [M+H – $H_2O$]$^+$, 362 [M+H – $C_2H_2O$]$^+$, 344, 261, 233, 144 |

LC-MS, liquid chromatography–mass spectrometry; LC-MS/MS, liquid chromatography–tandem mass spectrometry; [M+H]$^+$, protonated molecular ion.

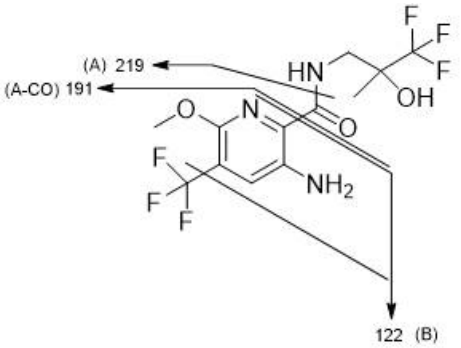
[a]The fragment was likely formed after neutral loss of anhydroglucuronic acid ($C_6H_8O_6$).

[b]The fragment was likely formed after loss of oxygen (O).

[c]The fragment was likely formed after loss of the $SO_3$ moiety.

[d]The fragment was likely formed after loss of the acetyl moiety.

*Nomenclature of fragment ions (icenticaftor as example):



**Supplemental Table 4.** Species comparison of biotransformation data of major metabolites following hepatocyte incubations.

| | Proportion of radioactivity (% of total) | | | |
|---|---|---|---|---|
| | Species | | | |
| Component | Mouse | Rat | Monkey | Human |
| P4.2 | – | 1.10 | – | – |
| P12.4 | – | 1.20 | – | – |
| M5 | 3.5 | 3.87 | – | 2.14 |
| M16 | – | 7.10 | – | – |
| M7 | – | 3.73 | – | – |
| P25.3 | – | 1.61 | – | – |
| P26.0 | – | – | – | – |
| M8 | 18.2 | 17.3 | 19.8 | 70.6 |
| M9 | – | 3.45 | 67.6 | 13.7 |
| M25 | – | – | – | 1.6 |
| P32.1 | – | – | – | 1.09 |
| M17 | – | 2.43 | – | – |
| Icenticaftor | 72.9 | 48.2 | 2.44 | 5.90 |
| Additional components | 3.0[a] | 5.13[a] | 2.14[a] | 1.91[a] |
| Total detected by HPLC | 97.6 | 95.1 | 92.0 | 97.0 |
| Pellet | 2.4 | 4.9 | 8.0 | 3.0 |

–, not detected; HPLC, high-performance liquid chromatography; P, peak with no assigned structure.

[a]No single additional component had >1.00% of total radioactivity.

Radiolabeled components following incubation of 10 μM [$^{14}$C]icenticaftor with hepatocytes derived from mouse, rat, monkey, and humans for up to 24 hours. The relative proportions of the parent compound and metabolites in the incubation were determined using HPLC analysis, with subsequent radioactivity detection.

**Supplemental Table 5.** Profiles of icenticaftor and its metabolites in bile duct-cannulated rats after the administration of a single intravenous dose of 3 mg/kg.

| | Excretion (% of dose) | | | |
|---|---|---|---|---|
| | Urine 0–48 hours | Feces 0–48 hours | Bile 0–48 hours | Total 0–48 hours |
| Front peak | 0.497 | 0.084 | 0.450 | 1.03 |
| M1 | 2.36 | – | 0.343 | 2.70 |
| P1.6 | 0.437 | – | 0.521 | 0.957 |
| M2 | 0.263 | 0.127 | 0.538 | 0.928 |
| P2.5 | 0.368 | – | – | 0.368 |
| M4 | 5.18 | – | 0.662 | 5.84 |
| P3.0–3.3 | 0.800 | 0.130 | 1.47 | 2.39 |
| M5 | 0.905 | 0.078 | 0.833 | 1.82 |
| P3.8 | 1.19 | 0.101 | 1.15 | 2.45 |
| M6 | 0.492 | 0.074 | 1.17 | 1.74 |
| M7 | 0.271 | 0.080 | 4.58 | 4.94 |
| P4.5–5.0 | 0.252 | 0.061 | – | 0.314 |
| **M8** | **4.10** | **0.021[a]** | **14.6** | **18.7** |
| **M9** | **4.16** | **0.016[a]** | **6.08** | **10.3** |
| M10 | 0.218 | 0.107 | 3.31 | 3.63 |
| P5.2–5.7 | 0.121 | – | 3.26 | 3.38 |
| M11 | 0.408 | 0.571 | 0.491 | 1.48 |
| P5.8 | – | 0.030 | 0.833 | 0.863 |
| M12 | 0.550 | 0.139 | 0.112 | 0.802 |
| P6.5–7.3 | 0.345 | 0.115 | 2.19 | 2.65 |

| | | | | |
|---|---|---|---|---|
| M13 | 0.076 | 0.165 | 0.408 | 0.652 |
| Icenticaftor | 1.56 | 0.761 | 12.1 | 14.4 |
| Sum of additional components | 1.75 | 0.141 | 4.05 | 5.94 |
| Total detected[b] | 26.3 | 2.80 | 59.1 | 88.2 |
| Lost during sample processing | 0.000 | 0.255 | 0.000 | 0.260 |
| RA in the pool analyzed | 26.3 | 3.05 | 59.1 | 88.5 |

–, not detected; P, peak with no assigned structure; RA, radioactive components; UPLC, ultra-performance liquid chromatography.

[a]Peaks were identified by retention time only.

[b]Total detected = total of all metabolites + icenticaftor + sum of additional components

Amounts (% of dose) of icenticaftor and its metabolites in the urine and feces of bile duct–cannulated male Han:WIST rats after the administration of a single intravenous dose of 3 mg/kg [$^{14}$C]icenticaftor (nominal dose). The concentrations of icenticaftor and its metabolites were determined from metabolic patterns obtained using UPLC analysis, with subsequent online radioactivity detection (pools of n = 3).

**Supplemental Table 6.** Profiles of icenticaftor and its metabolites in intact rats after the administration of a single oral dose of 10 mg/kg.

| | Excretion (% of dose) | | |
|---|---|---|---|
| | Urine | Feces | Total |
| Component | 0–96 h | 0–96 h | 0–96 h |
| Front peak | 0.992 | 2.27 | 3.26 |
| M1 | 7.75 | 0.139 | 7.89 |
| P1.6 | 0.52 | 0.466 | 0.986 |
| M2 | 0.663 | 0.314 | 0.978 |
| P2.5 | 2.47 | – | 2.47 |
| M4 | 9.11 | 1.05 | 10.2 |
| P3.0–3.3 | 2.29 | 4.54 | 6.83 |
| M5 | 1.78 | 3.77 | 5.56 |
| P3.8 | 1.94 | 2.18 | 4.12 |
| M6 | 0.958 | 1.76 | 2.72 |
| M7 | 0.121 | 1.18 | 1.30 |
| **M8** | **1.74** | **0.398** | **2.14** |
| **M9** | **0.682** | **1.13** | **1.81** |
| M10 | 0.211 | 0.366 | 0.577 |
| P5.2–5.7 | 1.31 | 2.83 | 4.13 |
| M11 | 0.467 | 3.08 | 3.55 |
| M12 | 0.63 | 0.812 | 1.44 |
| M13 | – | 0.939 | 0.939 |
| Icenticaftor | 0.49 | 9.19 | 9.68 |
| P8.8 | 1.07 | 0.617 | 1.69 |

| | | | |
|---|---|---|---|
| Sum of additional components | 2.5 | 2.75 | 5.25 |
| Total detected | 37.7 | 39.8 | 77.5 |
| Lost during sample processing | 0.000 | 2.90 | 2.90 |
| RA in the pool analyzed | 37.7 | 42.7 | 80.4 |

–, not detected; P, peak with no assigned structure; RA, radioactive components; UPLC, ultra-performance liquid chromatography.

[a]Peaks were identified by retention time only.

Amounts (% of dose) of icenticaftor and its metabolites excreted through urine and feces in male Han:WIST rats after the administration of a single oral dose of 10 mg/kg [$^{14}$C]icenticaftor (nominal dose). The concentrations of icenticaftor and its metabolites were determined from metabolic patterns obtained using UPLC analysis, with subsequent online radioactivity detection (pools of n = 3).

**Supplemental Table 7.** Kinetic parameters for icenticaftor glucuronidation in HLMs.

| Metabolite | $K_m$, (μM) (mean ± SD) | $V_{max}$ (pmol/min/mg) (mean ± SD) | $CL_{int}$ (μL/min/mg) (mean) | Relative contribution (%) (mean) |
|---|---|---|---|---|
| M8 | 10.3 ± 2.7 | 89.3 ± 8.0 | 8.67 | 76.1 |
| M9 | 7.18 ± 2.3 | 15.0 ± 1.5 | 2.09 | 18.3 |
| Total | 8.88 ± 1.7 | 101 ± 6.4 | 11.4 | 100 |

$CL_{int}$, intrinsic clearance; HLM, human liver microsome; $K_m$, Michaelis-Menten constant (substrate concentration producing half-maximal velocity); $V_{max}$, maximum velocity (reaction velocity at saturating substrate concentration).

**Supplemental Table 8.** Identification of human CYP and UGT isoenzymes involved in the metabolism of icenticaftor.

A. Recombinant enzyme kinetics

| Isoenzymes | Apparent $K_m$ (μM) (mean ± SD) | $K_m$ (μM)[b] | $V_{max}$ (pmol/min/mg) (mean ± SD) | $CL_{int,u}$ (μL/min/mg) (mean) |
|---|---|---|---|---|
| CYP1A2 | 7.43 ± 1.4 | 4.59 | 401 ± 41.4 | 87.4 |
| CYP3A4 | 19.4 ± 2.1 | 13.5 | 717 ± 59 | 53.1 |
| UGT1A8 | 277 ± 20 | 159 | 131 ± 17 | 0.824 |
| UGT1A9 | 0.593 ± 0.071 | 0.34 | 41.0 ± 1.1 | 121 |
| UGT2B7[a] | 8.09 ± 0.86 | 4.64 | 7.44 ± 0.24 | 1.60 |

$CL_{int}$, intrinsic clearance; $K_m$, Michaelis-Menten constant (substrate concentration producing half-maximal velocity); SD, standard deviation; UGT, uridine diphosphate glucuronosyltransferase; $V_{max}$, maximum velocity (reaction velocity at saturating substrate concentration).

[a]M9 formation rate. For other UGTs, the total metabolite formation rate was included in the kinetic analysis.

[b]$f_{u,mic}$ = 0.5 for UGT1A8, UGT1A9, and UGT2B7, 0.618 for CYP1A2, and 0.698 for CYP3A4.

B. Correlation analysis.

| | | Linear regression coefficient (R) | | |
|---|---|---|---|---|
| Marker reaction | Enzyme | M8 | M9 | Sum of all metabolites |
| 17β-Estradiol 3-glucuronidation | UGT1A1 | 0.502 | 0.180 | 0.505 |
| Trifluoperazine glucuronidation | UGT1A4 | 0.521 | 0.634 | 0.698 |

| | | | | |
|---|---|---|---|---|
| 1-Naphthol glucuronidation | UGT1A6 | 0.082 | 0.342 | 0.190 |
| Propofol glucuronidation | UGT1A9 | 0.519 | −0.212 | 0.415 |
| Morphine 3-glucuronidation | UGT2B7 | −0.150 | 0.920 | 0.184 |
| Morphine 6-glucuronidation | UGT2B7 | −0.122 | 0.899 | 0.206 |
| 17β-Estradiol 17β-glucuronidation | Unknown UGT | −0.185 | 0.607 | 0.053 |

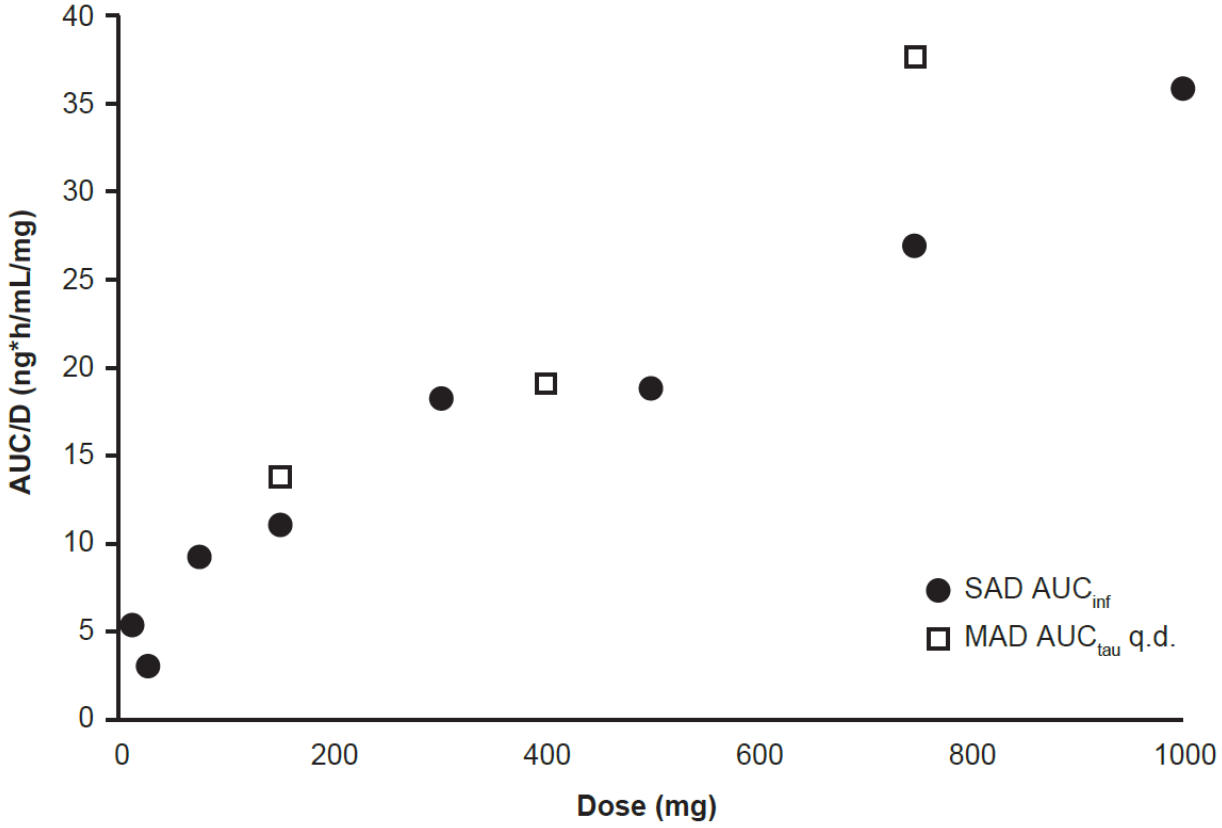
HLM, human liver microsome; UGT, uridine diphosphate glucuronosyltransferase.

HLMs (n = 16) were incubated with 3 μM [$^{14}$C]icenticaftor. The enzymatic reaction rates of different metabolic pathways were correlated with known activity of enzymes in the same bank of HLMs.

## 13. Supplemental Figures

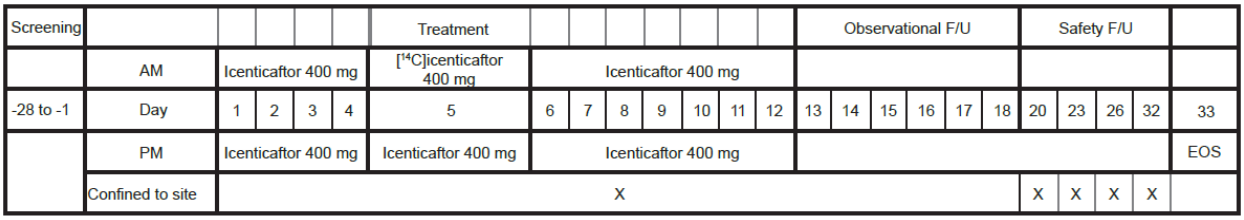
**Supplemental Fig. 1.** Dose nonlinearity after the administration of single and multiple-dose icenticaftor.

AUC/D, area under the curve per dose of icenticaftor; $AUC_{inf}$, area under the concentration-time curve from time zero to infinity; $AUC_{tau}$, area under the concentration-time curve from time zero to the end of the dosing interval.



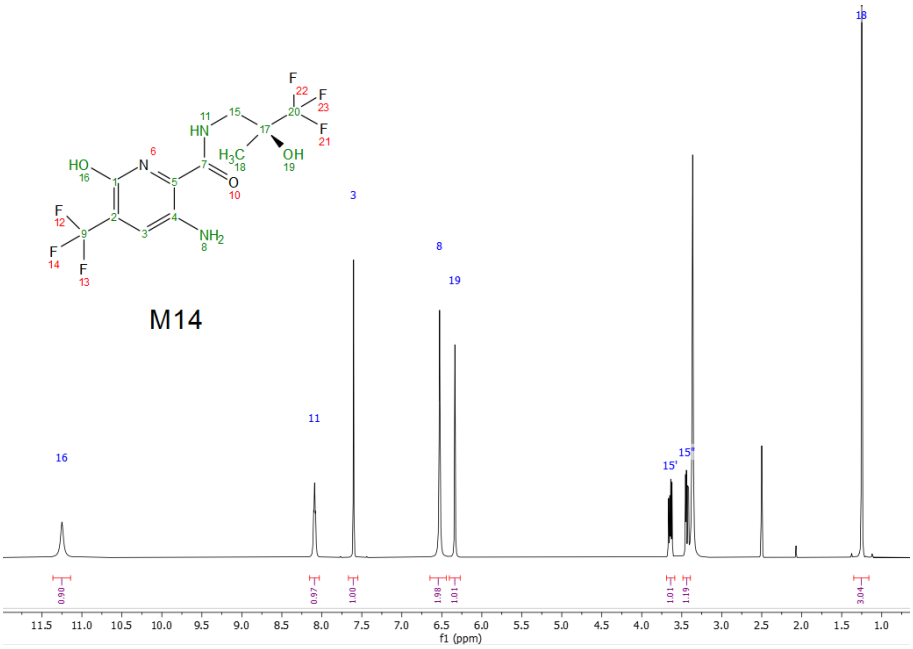
**Supplemental Fig. 2.** Study design.

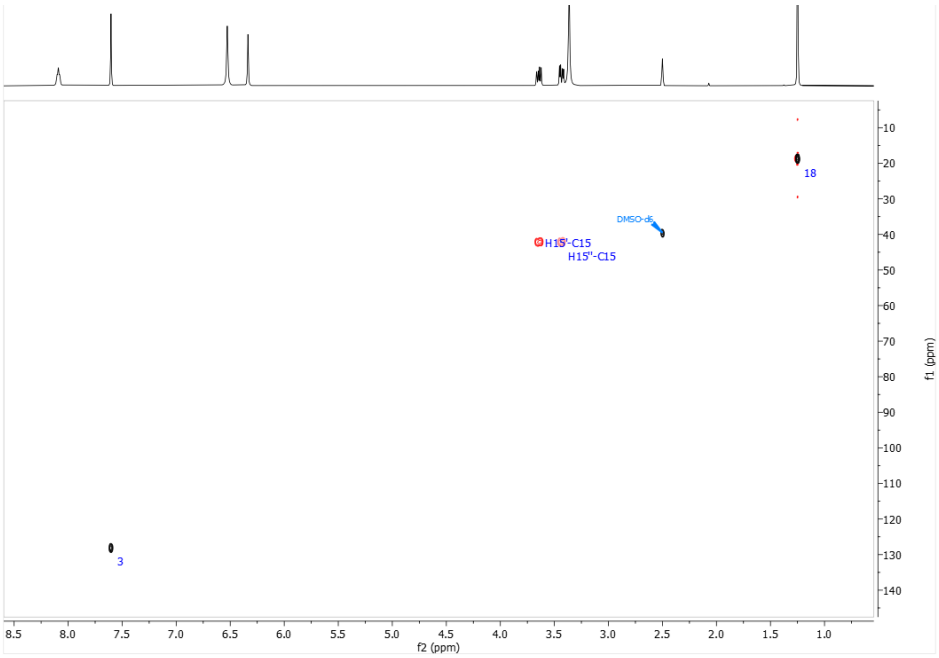
EOS, end of study; F/U, follow-up.



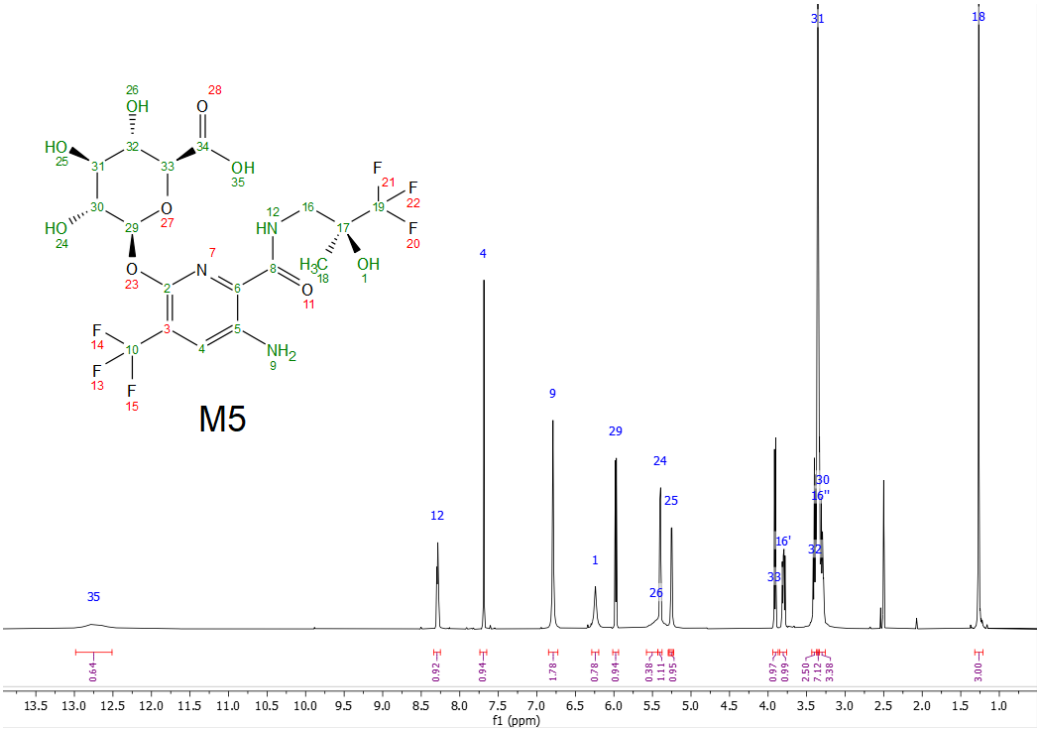
**Supplemental Fig. 3.** Characterization of reference standards by nuclear magnetic resonance spectroscopy. (A) Metabolite NIR205 (M14), (B) Metabolite IOD568 (M5), (C) Metabolite CKW231 (M8), (D) Metabolite CKW232 (M9).

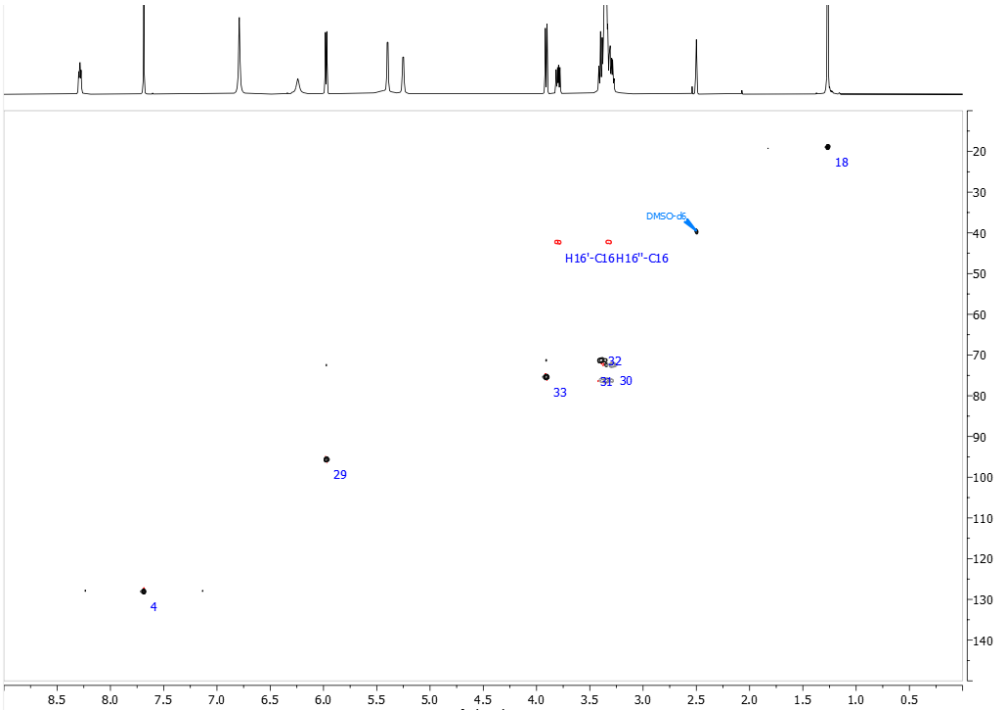
**A**



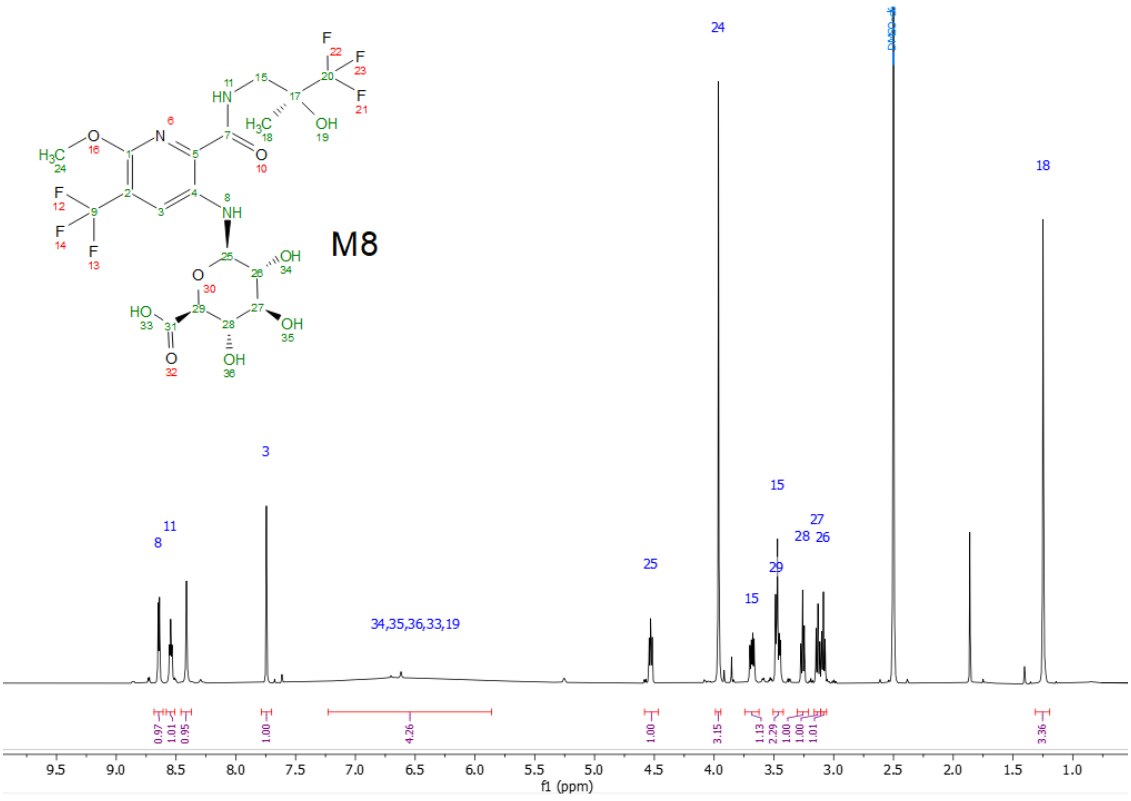



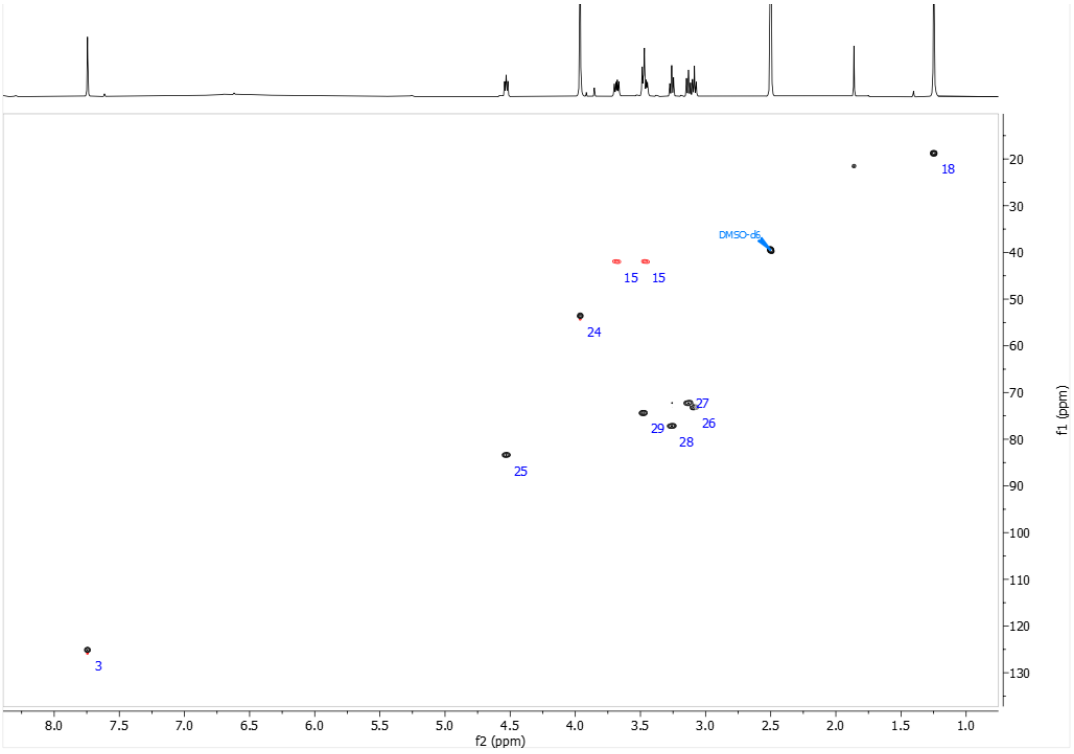
B



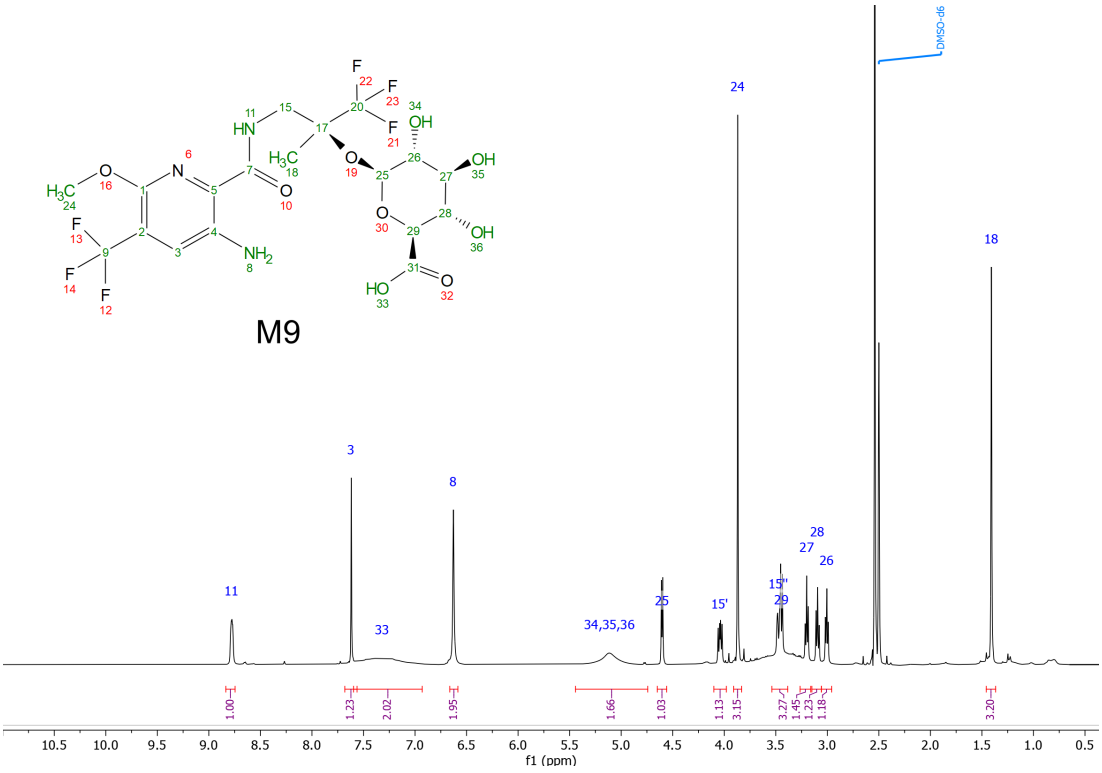



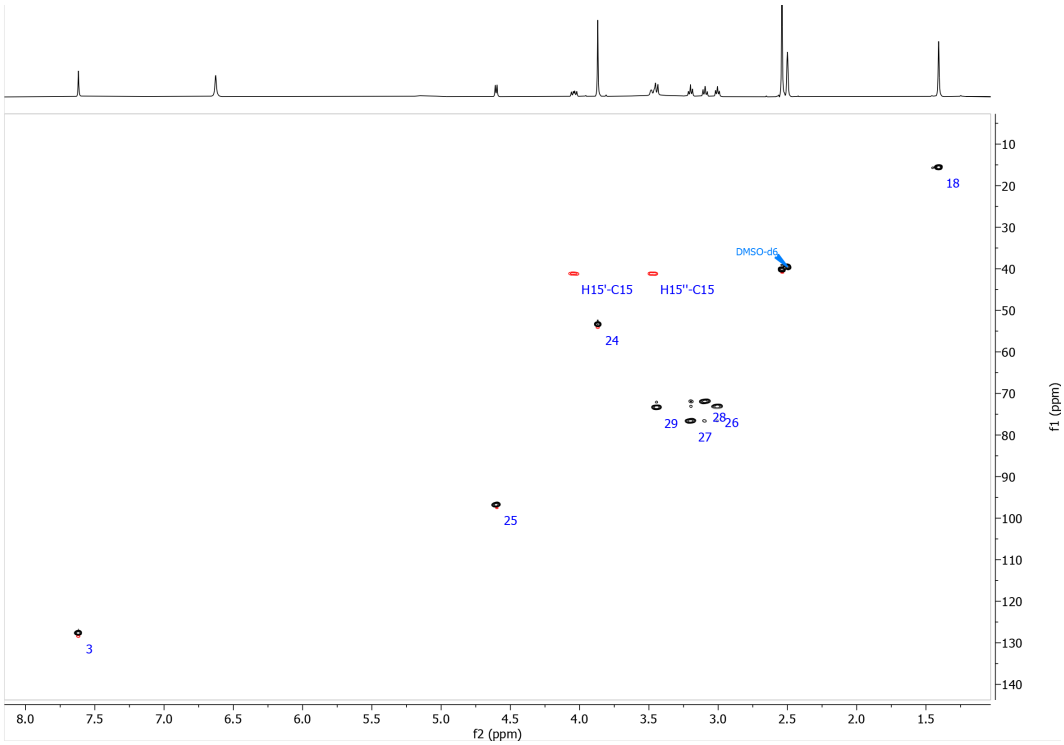
**C**


M8
24
18
3
11
8
34,35,36,33,19
25
15
29
15
27
28
26
f1 (ppm)

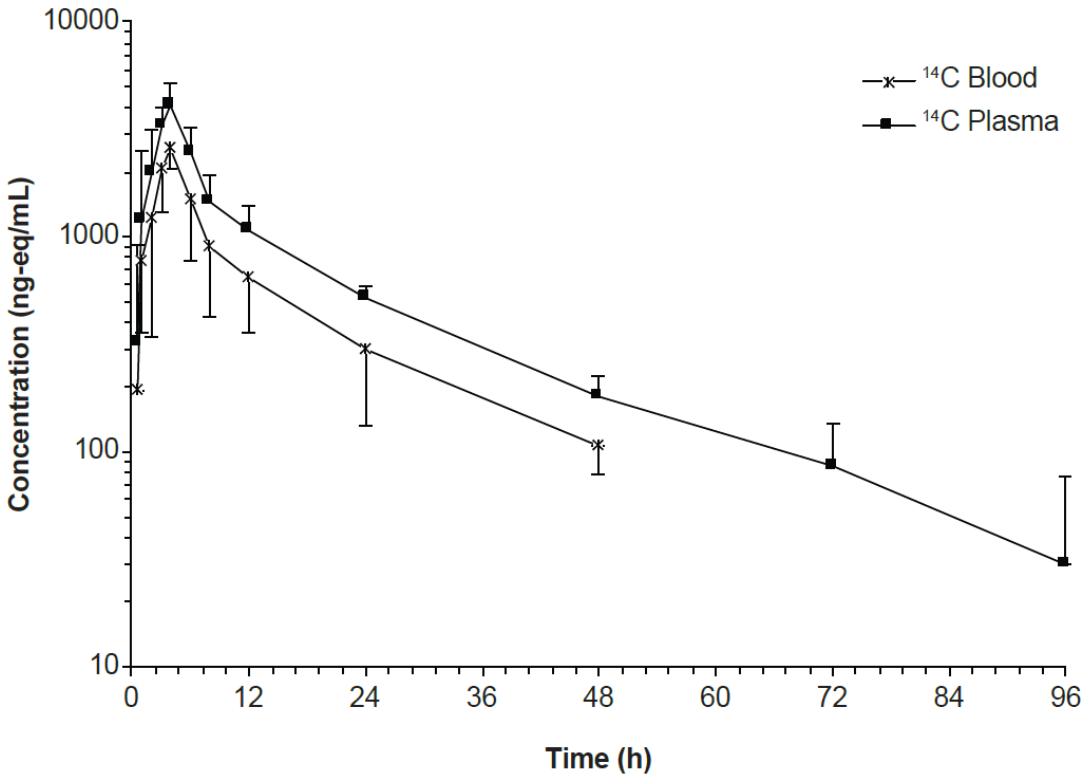

18
DMSO-d6
15
15
24
27
29
26
28
25
3
f2 (ppm)
f1 (ppm)

**D**


M9
DMSO-d6
24
18
3
8
28
27
26
11
33
34,35,36
25
15'
15''
29
1.00
1.23
2.02
1.95
1.66
1.03
1.13
3.15
3.27
1.45
1.23
1.18
3.20
f1 (ppm)


18
DMSO-d6
H15'-C15
H15''-C15
24
29
28
26
27
25
3
f2 (ppm)
f1 (ppm)

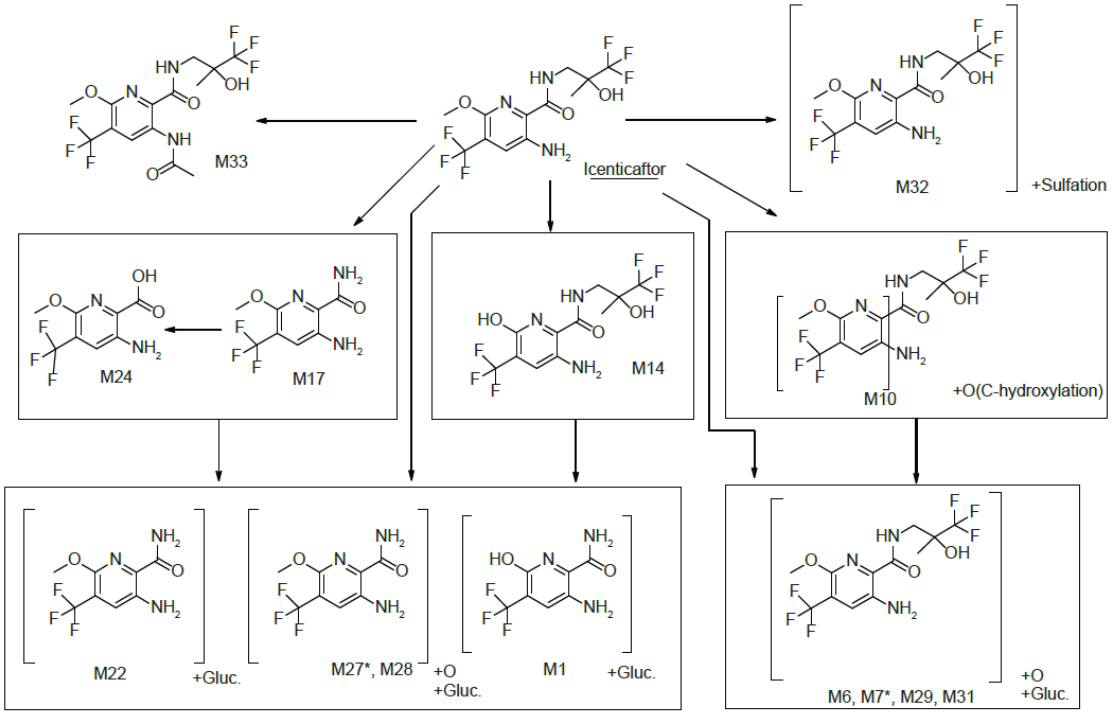
**Supplemental Fig. 4.** Total radioactivity concentrations in blood and plasma (semilogarithmic view).



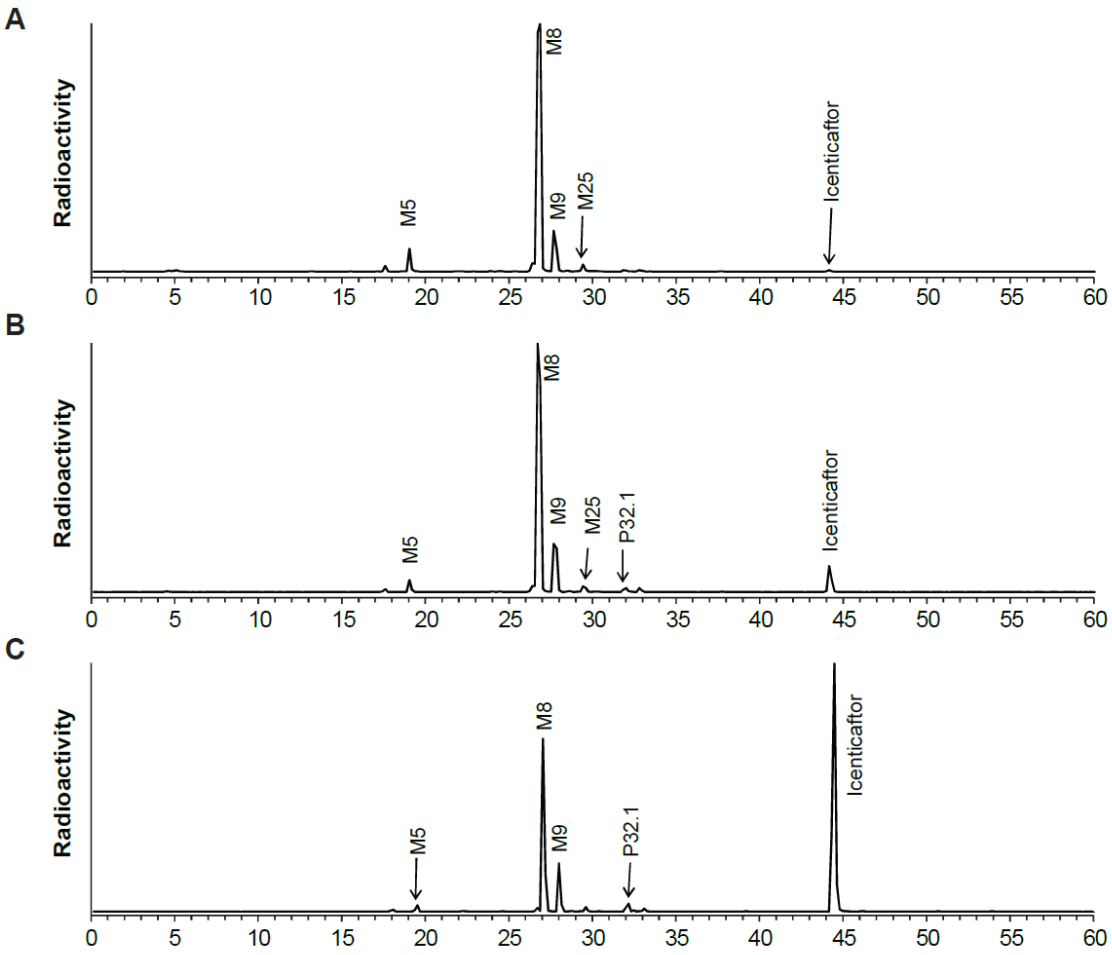
**Supplemental Fig. 5.** Scheme of the minor biotransformation pathways of icenticaftor in humans (Pathway II).

The metabolites were detected in plasma (p), urine (u), and/or feces (f).

*, N-oxygenation; Gluc, glucuronidation; +O, oxygenation.

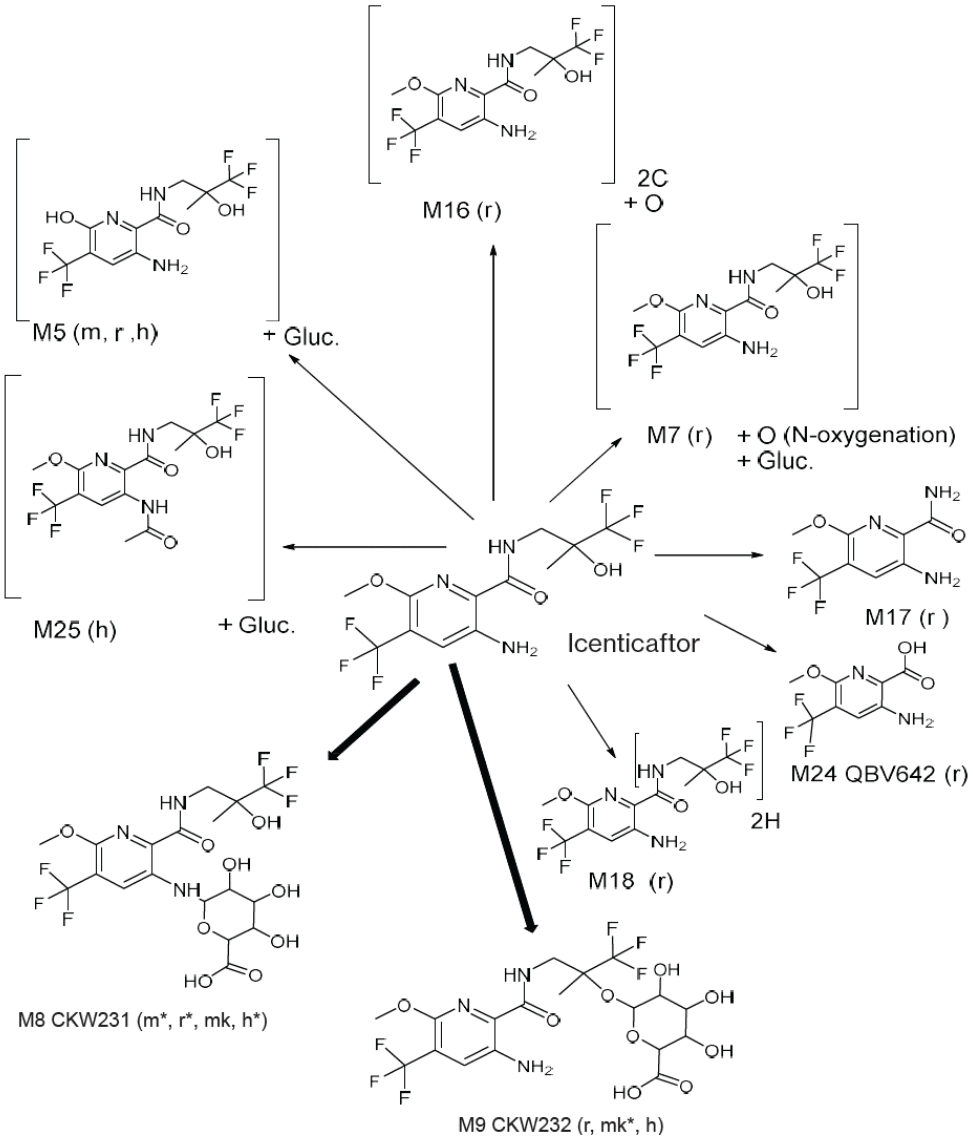


**Supplemental Fig. 6.** Metabolite profiles of [$^{14}$C]icenticaftor in human hepatocytes at concentrations of (A) 1 μM, (B) 10 μM, and (C) 30 μM at 24 hours.



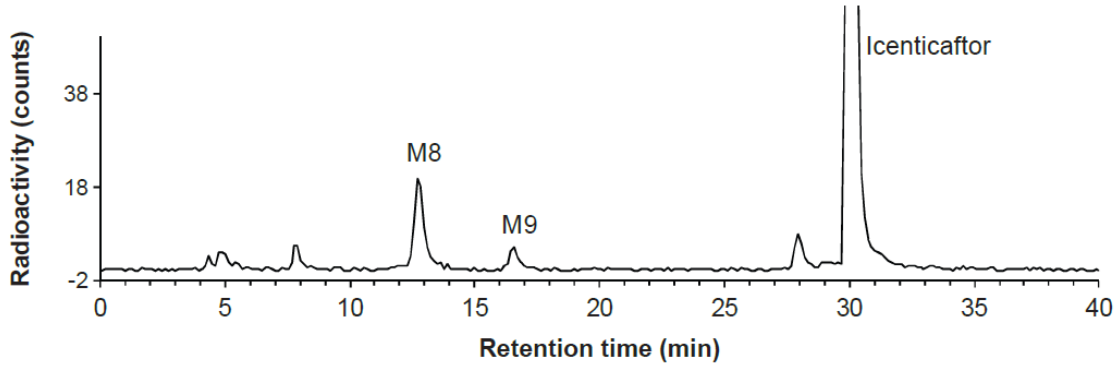
**Supplemental Fig. 7.** In vitro metabolic pathways across species.

*Major metabolite; Gluc, glucuronidation; h, human hepatocytes; m, mouse hepatocytes; mk, monkey hepatocytes; r, rat hepatocytes.



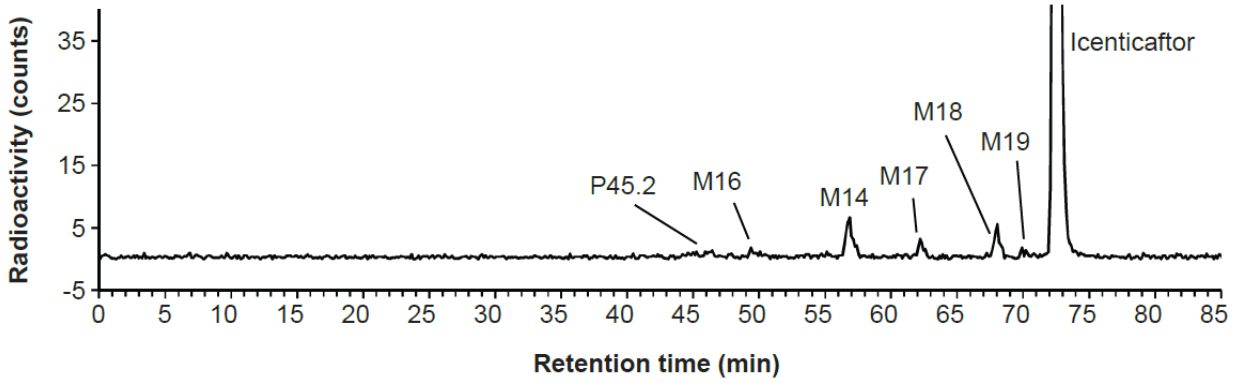
**Supplemental Fig. 8.** Radiochromatogram of [$^{14}$C]icenticaftor after incubation for 20 minutes in HLMs in the presence of UDPGA.

HLM, human liver microsome; UDPGA, uridine 5′-diphosphoglucuronic acid.



**Supplemental Fig. 9.** Radiochromatogram of icenticaftor after incubation in HLMs in the presence of NADPH.

HLM, human liver microsome; NADPH, nicotinamide adenine dinucleotide phosphate.



**Supplemental Fig. 10.** Unbound fraction ($f_{u,mic}$) of icenticaftor in HLMs.

Determination of the unbound fraction of 1 µM and 20 µM icenticaftor in microsomal incubations was performed in triplicates by ultracentrifugation. Values are presented as mean ± standard deviation of $f_{u,mic}$ for 1 µM and 20 µM icenticaftor. A trendline of the data points was calculated by fitting to a second-order polynomial function.

$f_{u,mic}$, unbound fraction; HLM, human liver microsome.