


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

Ulrike Glaenzel,  Felix Huth, Fabian Eggmann, Melissa Hackling, Luc Alexis Leuthold, Axel Meissner, and Lidiya Bebrevska

Novartis Pharma AG, Basel, Switzerland (U.G., F.H., F.E., L.A.L., A.M., L.B.) and Novartis Pharmaceuticals Corporation, East Hanover, New Jersey (M.H.)

Received April 9, 2024; Accepted September 9, 2024

ABSTRACT

Icenticaftor (QBW251) is a potentiator of the cystic fibrosis transmembrane conductance regulator protein and is currently in clinical development for the treatment of chronic obstructive pulmonary disease and chronic bronchitis. An absorption, distribution, metabolism, and excretion study was performed at steady state to determine the pharmacokinetics, mass balance, and metabolite profiles of icenticaftor in humans. In this open-label study, six healthy men were treated with unlabeled oral icenticaftor (400 mg b.i.d.) for 4 days. A single oral dose of [¹⁴C]icenticaftor was administered on Day 5, and unlabeled icenticaftor was administered twice daily from the evening of Day 5 to Day 12. Unchanged icenticaftor accounted for 18.5% of plasma radioactivity. Moderate to rapid absorption of icenticaftor was observed (median time to reach peak or maximum concentration: 4 hours), with 93.4% of the dose absorbed. It exhibited moderate distribution (V_z/F: 335 L) and was extensively metabolized, principally through N-glucuronidation, O-glucuronidation, and/or O-demethylation. The metabolites M8 and M9, formed by N-glucuronidation and O-glucuronidation of icenticaftor, respectively, represented the main entities detected in plasma (35.3% and 14.5%, respectively) in addition to unchanged icenticaftor (18.5%). The apparent mean terminal half-life of icenticaftor was 15.4 hours in blood and

20.6 hours in plasma. Icenticaftor was eliminated from the body mainly through metabolism followed by renal excretion, and excretion of radioactivity was complete after 9 days. In vitro phenotyping of icenticaftor showed that cytochrome P450 and uridine diphosphate glucuronosyltransferase were responsible for 31% and 69% of the total icenticaftor metabolism in human liver microsomes, respectively. This study provided invaluable insights into the disposition of icenticaftor.

SIGNIFICANCE STATEMENT

The absorption, distribution, metabolism, and excretion of a single radioactive oral dose of icenticaftor was evaluated at steady state to investigate the nonlinear pharmacokinetics observed previously with icenticaftor. [¹⁴C]icenticaftor demonstrated good systemic availability after oral administration and was extensively metabolized and moderately distributed to peripheral tissues. The most abundant metabolites, M8 and M9, were formed by N-glucuronidation and O-glucuronidation of icenticaftor, respectively. Phenotyping demonstrated that [¹⁴C]icenticaftor was metabolized predominantly by UGT1A9 with a remarkably low K_m value.

Introduction

Cystic fibrosis (CF) is a recessive genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator

(CFTR) gene (Hanssens et al., 2021). The CFTR protein is an adenosine triphosphate-binding transporter that plays a vital role in the transport of chloride and bicarbonate anions across the epithelial cell apical membrane of the lungs, pancreas, intestine, reproductive tract, and sweat glands, among others; it regulates salt absorption and is essential for the osmotic balance of mucus and its viscosity (Grand et al., 2021; Hanssens et al., 2021). CF and chronic obstructive pulmonary disease (COPD) are considered to be distinct diseases of unrelated origins; however, CFTR protein dysfunction commonly occurs in both conditions (Shi et al., 2018).

This study was funded by Novartis Pharma AG, Basel, Switzerland.

All authors are employees of Novartis. All authors except LB hold Novartis shares.

dx.doi.org/10.1124/dmd.124.001751.

 This article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: ADME, absorption, distribution, metabolism, and excretion; AE, adverse event; AUC_{%extrap}, percent of AUC_{inf} extrapolated; AUC, area under the curve; AUC_{inf}, area under the concentration-time curve from time zero to infinity; AUC_{last}, area under the concentration-time curve calculated from time zero to the last measured time point; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CL_{int,u}, unbound intrinsic clearance; CL_{ss}/F, apparent systemic (or total body) clearance from plasma following extravascular administration at steady state; C_{max}, maximum observed concentration; COPD, chronic obstructive pulmonary disease; CYP, cytochrome P450; FEV₁, forced expiratory volume in 1; second; f_m, fraction of the systemically available drug that is converted to metabolites; HPLC, high-performance liquid chromatography; HPLC-MS, high-performance liquid chromatography mass spectrometry; HLM, human liver microsome; K_m, Michaelis–Menten constant (substrate concentration producing half-maximal velocity); K_{m,u}, unbound Michaelis–Menten constant; LC-MS/MS, liquid chromatography tandem mass spectrometry; PK, pharmacokinetic; T_{1/2}, terminal half-life; T_{last}, time of last measured concentration; T_{max}, time to reach peak or maximum concentration; UGT, uridine diphosphate glucuronosyltransferase; V_{max}, maximum velocity (reaction velocity at saturating substrate concentration); V_z/F, apparent volume of distribution during the terminal elimination phase following extravascular administration; λ_z, terminal rate constant.

The flow of ions through activated CFTR channels can be increased by CFTR potentiators that enable effective opening of these channels (Van Goor et al., 2009; Grand et al., 2021). The CFTR potentiator ivacaftor has been shown to provide clinical and physiological improvements in patients with CF and is approved for the treatment of patients with CF who have specific CFTR mutations (Ramsey et al., 2011; US Food and Drug Administration, 2012; European Medicines Agency, 2012a; Rowe et al., 2014; Moss et al., 2015). Icatibafator (QBW251) is a new, orally bioavailable, low-molecular-weight CFTR potentiator of both the wild-type and mutated/defective forms of the CFTR protein that has been investigated in patients with CF and COPD (Rowe et al., 2020; Kazani et al., 2021; Mall et al., 2023; Martinez et al., 2023). Icatibafator up to 750 mg twice daily was well tolerated in the first-in-human study of healthy subjects and up to 450 mg twice daily in patients with CF (Kazani et al., 2021). Moderate to rapid absorption of icatibafator (time to reach peak or maximum concentration $[T_{max}]$, 0.8–4 hours) was observed, and the overall terminal half-life ($T_{1/2}$) was 10–13 hours in healthy subjects. The mean maximum observed concentration (C_{max}) in patients with CF ranged from 419 ng/ml (150 mg b.i.d., Day 1) to 4080 ng/ml (450 mg b.i.d., Day 14). Icatibafator is currently in development as an adjunctive therapy for the treatment of COPD and chronic bronchitis. In a Phase 2 proof-of-concept study conducted in patients with COPD, improvements in respiratory function (pre- and postbronchodilator forced expiratory volume in 1 second [FEV₁]) were observed over 4 weeks of icatibafator treatment (Rowe et al., 2020). In addition, the results of a Phase 2b dose-finding study with icatibafator demonstrated potentially clinically relevant benefits for patients with COPD and chronic bronchitis, including improvement in trough FEV₁, reduction in cough, sputum, and rescue medication use, and a decline in fibrinogen levels at 24 weeks (Martinez et al., 2023).

The metabolic characteristics of icatibafator have not been reported previously. Human absorption, distribution, metabolism, and excretion (ADME) studies are essential for understanding the pharmacokinetic (PK) properties of pharmaceutical drug candidates within the body, including the processing of a drug and its metabolites and their impact on drug safety (Coppola et al., 2019; Lindmark et al., 2023).

This study was designed to evaluate the ADME properties of icatibafator, including PK parameters, in healthy volunteers. In addition, it was supplemented with an *in vivo* rat ADME study and *in vitro* assays with mouse, rat, monkey, and human hepatocytes for better elucidation of the clearance pathways and enzymes involved in the metabolism of icatibafator.

Materials and Methods

Study Rationale

This single-center, open-label study investigated the ADME of icatibafator, which has been shown to have nonlinear exposure with increasing dose after both single- and multiple-dose administration (Supplemental Fig. 1). The European Medicines Agency guidelines assert that a single-dose study is sufficient to investigate the ADME properties of a drug if there is no dose or time dependency in the first-pass metabolism or elimination of the drug (European Medicines Agency, 2012b). These guidelines also state that if the elimination is nonlinear, the design and degree of saturation should mimic the therapeutic situation for nonlinear elimination, that is, for drugs with dose-dependent elimination and significant accumulation under multiple dosing, a single dose of the radiolabeled drug should be administered when steady state has been reached with nonradiolabeled drug (European Medicines Agency, 2012b). Similarly, the United States (US) Food and Drug Administration guidelines state that a single-dose mass balance study is generally sufficient (US Food and Drug Administration, 2024). Under certain instances, a multiple-dose study can be considered, in which subjects would receive a single dose of radiolabeled drug after reaching steady state conditions with the nonradiolabeled drug; however, this approach evaluates only the clearance pathway of the radiolabeled drug, whereas bioanalysis of the nonradiolabeled moieties at steady

state can be useful for interpreting the results (US Food and Drug Administration, 2024). Therefore, to satisfy agency guidelines, the present human ADME study was conducted at steady state with a single, oral, radiolabeled dose of icatibafator.

In vitro investigations were also conducted to identify the metabolizing enzymes involved in the clearance of icatibafator. Early biotransformation data indicated the involvement of glucuronidation and oxidative metabolism; therefore, human cytochrome P450 (CYP) and uridine diphosphate glucuronosyltransferase (UGT) phenotyping was performed.

Study Design and Subjects

This Phase 1, single-center, open-label study was conducted in six healthy male volunteers. Subjects were 18–55 years old, with a body mass index of 18.0–30.0 kg/m² and body weight of 55–120 kg. Other key inclusion and exclusion criteria are detailed in the Supplemental Material.

The study included a 28-day screening period (including baseline period of 1 day) followed by a 12-day treatment period (see Supplemental Fig. 2). Nonradiolabeled icatibafator (400 mg) was administered twice daily for the first 4 days (Days 1–4). On Day 5, at steady state, a single dose of [¹⁴C]icatibafator 3.7 MBq (100 μCi) 400 mg was administered in the morning followed by a single dose of nonradiolabeled icatibafator 400 mg in the evening. Further doses of nonradiolabeled icatibafator (400 mg b.i.d.) were given on Days 6–12. All treatment administrations occurred approximately 30 minutes after consumption of a standardized meal.

Ethics Approval

The study was conducted in accordance with the ethical principles consistent with the International Conference on Harmonization Good Clinical Practice guidance E6, and the protocol was reviewed and approved by the independent ethics committee at the study site. All subjects provided written informed consent.

Study Objectives

The primary objectives of the study were to (1) determine the rates and routes of excretion of [¹⁴C]icatibafator-related radioactivity, including mass balance of total drug-related radioactivity in urine and feces following a single 400 mg oral dose of [¹⁴C]icatibafator at steady state; (2) determine the PK of total radioactivity in blood and plasma; and (3) characterize the plasma PK of icatibafator. The secondary objective was to assess the safety and tolerability of multiple oral doses of 400 mg of icatibafator. Exploratory objectives included the identification and semiquantification of icatibafator and its metabolites in plasma and excreta (urine and feces) to elucidate key biotransformation pathways and clearance mechanisms in humans and to characterize the plasma PK of icatibafator and its key metabolite(s), based on radiometry data.

Study Treatments

The 400 mg dose containing 3.7 MBq [¹⁴C]-radiolabeled icatibafator was chosen to provide sufficiently high drug and metabolite levels to meet the objectives of this study without safety concerns. The radiation exposure for a subject had been estimated according to the International Commission on Radiological Protection and was considered to be acceptable (<1 mSv). Nonradiolabeled icatibafator was provided as 100 mg hard gelatin capsules (Novartis Pharma AG, Basel, Switzerland), and the molecular weight of the nonradiolabeled, free base compound was 361.24 g/mol. [¹⁴C]icatibafator was supplied as 100 mg hard gelatin capsules (Almac Sciences, Ltd., Craigavon, UK). Radiochemical purity was

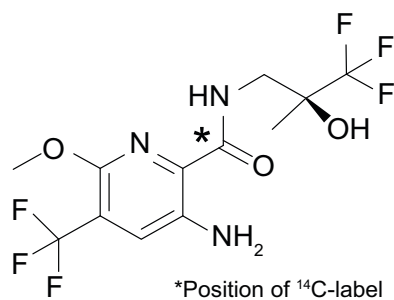


Fig. 1. Chemical structure of the radiolabeled icatibafator.

99.7%. The actual specific radioactivity of the solid drug substance was 9.15 kBq/mg (free base) and was measured at Almac Sciences, Ltd. (Craigavon, UK). The bottles containing the capsules and outer packaging were individually labeled with details of the investigational agent. The chemical structure of the compound, including the position of the radiolabel, is shown in Fig. 1.

Chemicals and Standards

Nonradiolabeled icenticaftor was synthesized and provided by Novartis Pharma AG (Basel, Switzerland). Radiolabeling of icenticaftor ($[^{14}\text{C}]$ icenticaftor) was performed by Almac Sciences Ltd. (Craigavon, UK) and $[^{13}\text{CD}_3]$ icenticaftor (internal standard) was supplied by Tjaden Biosciences (Iowa, USA). Reference standards for the metabolites M8, M9, and M14 were synthesized and provided by Novartis Pharma AG (Basel, Switzerland), and the standard for M5 was provided by Hypha Discovery Ltd. (Abingdon, UK), as detailed in the Supplemental Materials, Section 6. Other chemicals and solvents were obtained from commercial sources and were of analytical grade.

Sample Collection and Aliquoting

Blood and plasma samples. Blood samples were collected predose on Days -1 and 5 and then at certain time points (0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192, and 216 hours) after the administration of the $[^{14}\text{C}]$ icenticaftor dose on Day 5. All blood samples (8 ml or 18 ml) were collected either by direct venipuncture or using an indwelling cannula inserted in a forearm vein. Aliquots were collected for radioactivity determination and metabolite analysis.

Urine samples. Predose (blank) samples were collected from each subject on Day -1 . After the administration of the radiolabeled dose on Day 5, all urine samples were collected during the time intervals 0–6, 6–12, and 12–24 hours and, thereafter, in 24-hour fractions up to 216 hours. To stabilize the acid-labile N-glucuronide in the individual urine fractions, the pH was adjusted to neutral pH values. Aliquots were collected for radioactivity determination (two aliquots of 1 ml) and metabolite analysis (two aliquots of 20 ml). The urine samples were frozen and stored below -60°C until analysis.

Fecal samples. Predose (blank) samples were collected from each subject on Day -1 . Following the administration of the radiolabeled dose on Day 5, all fecal samples were collected and pooled per 24 hours, if needed, during the post-dose sample collection period of 216 hours. Each sample was diluted with one to two volumes of water containing a suitable suspension stabilizer and then homogenized. Aliquots were collected for radioactivity determination and metabolite analysis.

Full details of the sample collection schedule, storage conditions and processing/analytical facilities are provided in the Supplemental Material.

Analysis of Total Radioactivity

Total $[^{14}\text{C}]$ concentrations of icenticaftor and its metabolites were determined using liquid scintillation counting or combustion with subsequent liquid scintillation counting (Supplemental Material).

Determination of Plasma Icenticaftor Concentrations

Plasma concentrations of unchanged icenticaftor were measured using a validated liquid chromatography tandem mass spectrometry (LC-MS/MS) assay (Supplemental Material).

Determination of Metabolite Profiles in Plasma, Urine, and Feces

Metabolite profiles in plasma, urine, and feces were investigated for up to 48, 96, and 120 hours, respectively. Plasma samples were pooled by combining identical aliquots of the same timepoints (time pools). Extracts of plasma samples were obtained by protein precipitation with acetonitrile. The final extracts were evaporated, reconstituted, and analyzed using high-performance liquid chromatography mass spectrometry (HPLC-MS).

Urine samples collected from each subject and pooled across 0–96 hours were prepared by combining identical volume percentages of the different urine fractions. A 150 μl aliquot was used for HPLC-MS analysis (Supplemental Material).

Fecal samples collected from each subject were pooled across 0–120 hours, and a homogenate pool prepared by combining identical volume percentages of the different homogenate fractions. Aliquots of pooled feces reconstituted sample extracts (100 μl) were analyzed by HPLC-MS using the Agilent model 1200

HPLC system (Agilent Technologies, Waldbronn, Germany). The detailed methods are described in the Supplemental Material.

Structural Characterization of Metabolites

Structures of metabolites in plasma and excreta were characterized using LC-MS/MS analysis. Offline radioactivity detection was used to correlate peaks with mass spectral data. Product ion mass spectra, exact mass measurement, and hydrogen/deuterium exchange experiments were used to derive metabolite structures, which were compared with those of synthetic standards where possible. Synthetic reference standards were prepared using biosynthetic methods (Supplemental Material, Section 6) and characterized by nuclear magnetic resonance spectroscopy (Supplemental Fig. 3).

Data Analysis

Multiple-dose plasma PK parameters C_{max} , T_{max} , area under the concentration-time curve calculated from time zero to the last measured time point $[\text{AUC}_{\text{last}}]$, apparent volume of distribution during the terminal elimination phase following extravascular administration $[\text{Vz}/\text{F}]$, apparent systemic [or total body] clearance from plasma following extravascular administration at steady state $[\text{CL}_{\text{ss}}/\text{F}]$ for icenticaftor and its metabolites at steady state were derived from plasma concentration versus time data. Single-dose blood and plasma PK parameters (C_{max} , T_{max} , $T_{1/2}$, AUC_{last} , and area under the concentration-time curve from time zero to infinity $[\text{AUC}_{\text{inf}}]$) for total radioactivity were derived from plasma and blood concentration versus time data for total radioactivity.

PK calculations were based on the recorded time of sample collection, and PK parameters were calculated using noncompartmental analysis (WinNonlin version 6.4; Certara, Princeton, NJ, USA). The terminal rate constant (λ_z) was determined from linear regression of at least the last three, nonzero measurements in the terminal phase of the log-transformed concentration-time profile (WinNonlin algorithm). The extent of icenticaftor and metabolite excretion in urine and feces was expressed as a percentage of the total radioactive dose administered.

The fraction of radioactivity in plasma was calculated: Fraction of plasma radioactivity (%) = (plasma radioactivity concentration/blood radioactivity concentration) \times (1 – hematocrit) \times 100.

In Vitro Phenotyping of Icenticaftor Metabolites

In vitro phenotyping was conducted to quantitatively evaluate the contribution of human CYP and UGT enzymes in the metabolism of $[^{14}\text{C}]$ icenticaftor in human liver microsomes (HLMs) and recombinant enzymes (Supplemental Material).

In Vitro Biotransformation in Mouse, Rat, Monkey, and Human Hepatocytes

$[^{14}\text{C}]$ icenticaftor was incubated with hepatocytes derived from male ICR/CD-1 mice, male Sprague Dawley rats, male cynomolgus monkeys, and humans (both males and for up to 24 hours). Metabolites were extracted and analyzed by HPLC, with offline radioactivity detection. Metabolite structures were characterized using liquid chromatography mass spectrometry with accurate mass or LC-MS/MS and deuterium exchange. The detailed methods are described in the Supplemental Material.

Rat ADME Study

The ADME of icenticaftor in male Wistar rats (Han:WIST, albino) was assessed after the administration of a single intravenous dose (3 mg/kg) or oral administration (10 mg/kg) of $[^{14}\text{C}]$ icenticaftor. The detailed methods are described in the Supplemental Material.

Assessment of Safety

All adverse events (AEs), serious AEs, vital signs, laboratory evaluations (hematology, clinical chemistry, and urinalysis), and electrocardiogram were monitored and recorded.

Statistical Analysis

No inferential statistical analysis was performed. Descriptive statistics are provided.

TABLE 1
Primary pharmacokinetic parameters of radioactivity and icenticaftor in blood and plasma

PK parameter (units) ^a	Total blood radioactivity	Total plasma radioactivity	Plasma icenticaftor
T _{max} (h), median (range)	4 (1–4)	4 (1–4)	4 (1–4)
C _{max} (ng/ml)	2670 (509)	4280 (838)	1680 (568)
T _{last} (h), range	24–48	48–96	12
AUC _{last} (ng*h/ml)	24700 (5290)	45600 (7830)	8170 (3270)
T _{1/2} (h)	15.4 (5.79)	20.6 (9.55)	–
Typical time interval (h), range ^b	8–48	24–96	–
AUC _{inf} (ng*h/ml)	27900 (4610)	48300 (8120)	–
Percentage of [¹⁴ C]-AUC _{inf} plasma	57.9 (2.91)	–	16.6 (5.06)
AUC%extrap	12.2 (6.21)	5.74 (2.53)	N/A
Vz/F (L)	–	–	335 (161)
CL _{ss} /F (L/h)	–	–	56.8 (25.6)

–, not calculated; AUC%extrap, percentage of AUC_{inf} extrapolated; N/A, not applicable; T_{last}, time of last measured concentration.

^a Mean (S.D.) unless otherwise stated.

^b The range shows the time interval for the elimination half-life, which highlights the differences between blood and plasma. A shorter half-life in blood is likely to be caused by a higher lower limit of quantification than that in plasma.

Results

Subject Demographics

All six male subjects completed the study. Their median age was 32.0 years (range: 23–55 years), and the median body mass index was 26.34 kg/m² (range: 21.39–29.41 kg/m²).

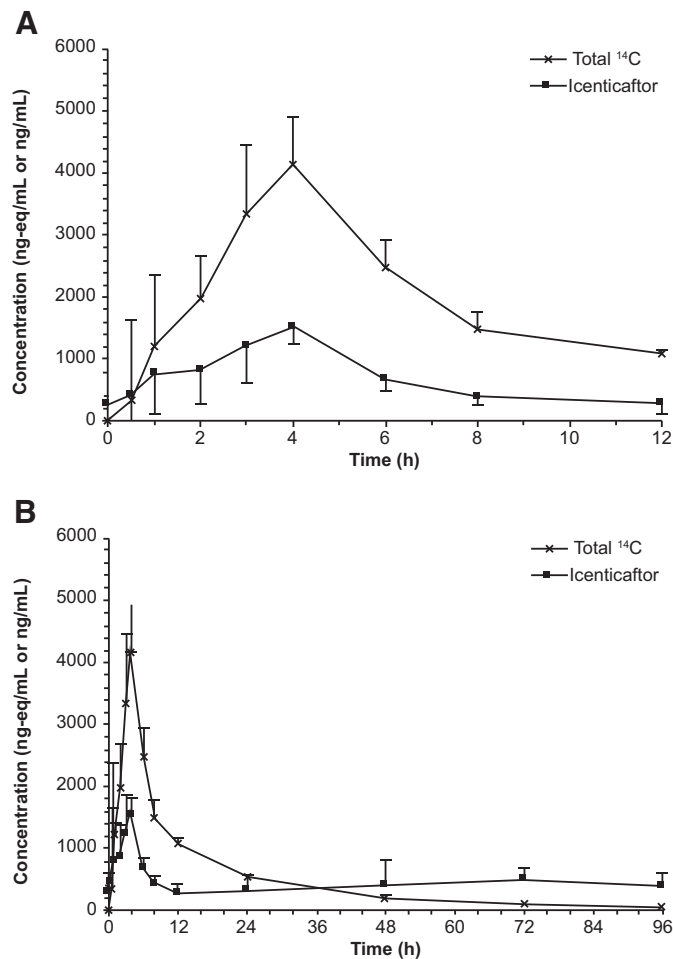


Fig. 2. Arithmetic mean (S.D.) plasma concentrations of total radioactivity and icenticaftor. (A) Linear view 0–12 hours after the administration of the [¹⁴C]icenticaftor dose. (B) Linear view 0–96 hours after the [¹⁴C]icenticaftor dose. The figures show a combination of radioactive (liquid scintillation counting) and non-radioactive (LC-MS/MS) methods. The radiolabeled drug-related compounds (total ¹⁴C) display single-dose PK, whereas the icenticaftor concentrations reflect steady state PK.

Absorption and Concentration of Radioactivity and Icenticaftor in Plasma or Blood

Icenticaftor demonstrated moderate to rapid absorption following oral administration, with a median T_{max} of 4 hours (range: 1–4 hours) and 93.4% of the oral dose absorbed. Exposure to icenticaftor relative to total radioactivity was 16.6% of radioactivity AUC_{inf} (Table 1). Arithmetic mean plasma concentrations of total radioactivity and icenticaftor are shown in Fig. 2. After oral administration of [¹⁴C]icenticaftor at steady state, radioactivity was detected in blood and plasma for up to 48 hours and 96 hours postdose, respectively; thereafter, radioactivity levels were below the limit of quantification (Supplemental Fig. 4).

The PK parameters of icenticaftor in plasma at steady state (Table 1) showed moderate intersubject variability for C_{max} (33.8%) and AUC_{last} (40%). The mean values of C_{max} and AUC_{last} were 1680 ng/ml and 8170 ng*h/ml, respectively. The individual C_{max} and AUC_{last} (0–12 hours) values ranged from 912 to 2360 ng/ml and from 4240 to 12900 ng*h/ml, respectively.

Distribution of Icenticaftor

The mean Vz/F of icenticaftor in plasma was 335 L (Table 1), with a mean coefficient of variation percentage of 48.1%.

The mean blood/plasma AUC_{inf} ratio of radioactivity was 0.579 (range: 0.542–0.620). Additionally, icenticaftor and/or its metabolites did not exhibit any special affinity to erythrocytes as indicated by the similar ratio and decline of compound-related radioactivity in blood and plasma in the concentration-time curves (Supplemental Fig. 4). Compared with blood, the mean ± S.D. fraction of total radioactivity in plasma was 93.3 ± 3.35%.

Metabolism of Icenticaftor

Icenticaftor was extensively metabolized mainly by N-glucuronidation, O-glucuronidation, and/or O-demethylation (Table 2). The concentrations of icenticaftor and metabolites in plasma following a single oral dose of 400 mg [¹⁴C]icenticaftor at steady state, and the estimated AUCs are presented in Supplemental Table 1. Unchanged icenticaftor (radioactivity AUC_{0–48h} of 18.5%) and its metabolites, M8 (AUC_{0–48h} of 35.3%) and M9 (AUC_{0–48h} of 14.5%), represented the main circulating entities in plasma (Table 2). M8 and M9 were formed by N-glucuronidation and O-glucuronidation of icenticaftor, respectively. Another metabolite, M5 (formed by O-demethylation and glucuronidation), accounted for 10.7% of the plasma [¹⁴C]-AUC_{0–48h}. Other minor metabolites were identified but each accounted for ≤3% of the plasma [¹⁴C]-AUC_{0–48h} (Supplemental Table 1). Figure 3 shows a representative metabolite profile in plasma at 4 hours and 48 hours postdose, and Fig. 4 shows the

TABLE 2
AUC_{0–48h}, AUC_{inf}, and T_{1/2} of icenticaftor and its metabolites in plasma based on metabolite pattern analysis

Peak ^a	Compound/ metabolite	AUC _{0–48h} (nmol*h/L)	Percentage AUC _{0–48h}	AUC _{inf} (nmol*h/L)	Percentage AUC _{inf}	T _{1/2} (h)
M5	O-demethylation, glucuronidation	12800	10.7	13800	10.7	12.1
M8	N-glucuronidation	42200	35.3	44100	33.9	11.2
M9	O-glucuronidation	17300	14.5	18100	13.9	11.6
M10	C-hydroxylation	2310	1.93	2840	2.19	–
M14	O-demethylation	1790	1.50	2000	1.54	16.8
M17	N-dealkylation	3630	3.04	3840	2.96	10.7
Icenticaftor	Parent drug	22100	18.5	22700 ^b	17.5	11.2
	Sum of minor identified metabolites (each ≤1%)	7930	6.63	–	–	–
	Sum of unknown trace metabolites (each ≤0.30%)	515	0.430	–	–	–
	Lost during sample processing and HPLC	8870	7.42	–	–	–
Total ¹⁴ C (total of radiolabeled components)		120000	100	130000	100	14.2

–, not calculated; AUC_{0–48h}, area under the concentration-time curve from time zero to 48 hours.

^aListed in order of elution.

^b8200 ng*h/ml.

concentration-time course of icenticaftor and its main metabolites in plasma at 0–48 hours postdose.

Metabolites were excreted mainly in urine, with M8, M9, and M5 being the most abundant metabolites (50.1%, 18.4%, and 11.0% of the radioactive dose, respectively; Fig. 5 and Supplemental Table 2). Other metabolites in urine, most of which contributed to ≤1% of the radioactive dose, were mainly formed by oxygenation, N-oxidation, O-demethylation, N-dealkylation and/or glucuronidation(s). Unchanged icenticaftor was the main component in feces (2.68% of the radioactive dose; Fig. 5 and Supplemental Table 2). Minor metabolites detected in feces included M14, M32, and M33, which were formed by O-demethylation, sulfation, and N-acetylation, respectively, of the parent drug. The structures of the metabolites were derived from LC-MS/MS analysis; mass spectral data showing the order of elution and major signals are presented in Supplemental Table 3 and the electrospray mass spectrum of icenticaftor is shown in Fig. 6. Based on all the information gained, the proposed major biotransformation pathway of icenticaftor is depicted in Fig. 7. The proposed minor biotransformation pathway of icenticaftor is shown in Supplemental Fig. 5. Overall, icenticaftor was mainly metabolized by N-glucuronidation, O-glucuronidation, and/or O-demethylation, forming the metabolites M8, M9, and M5; however, N-dealkylation, N-oxidation, oxygenation, C-hydroxylation, formation of a carboxylic acid, sulfation, and N-acetylation also contributed to a minor extent to the biotransformation of icenticaftor.

In vitro biotransformation data from mouse, rat, monkey, and human hepatocytes indicated that the major metabolites (above 5% of the total radioactivity) were M8 (all four species), M9 (monkey and human), and M16 (rat) (Supplemental Table 4). The main metabolite in mouse, rat, and human hepatocytes was M8 (direct N-glucuronide) and in monkey hepatocytes, M9 (O-glucuronide); however, different profiles for minor metabolites were observed in the different species. A minor metabolic pathway in mouse, rat, and human hepatocytes involved O-demethylation on the pyridine moiety and subsequent glucuronidation (M5). Supplemental Fig. 6 shows the metabolic profiles in human hepatocytes at concentrations of 1–30 μM, with major metabolites M8 and M9 as well as some minor metabolites (e.g., M5 and M25). Supplemental Fig. 7 shows the in vitro metabolic pathways observed across species as described above and in Supplemental Table 4. In vivo metabolic profiles obtained in rats showed that M8 and M9 were the main components in the bile of bile duct-cannulated rats (Supplemental Table 5), while only trace levels were present in feces of intact rats (Supplemental Table 6). Additionally, the larger amount of unchanged drug in the feces of intact rats (9.19% of the dose) indicates hydrolysis of the glucuronides in the intestine of rats by intestinal microbiota. These in vivo data are in line with the in vitro data for metabolism following hepatocyte incubations (Supplemental Table 4).

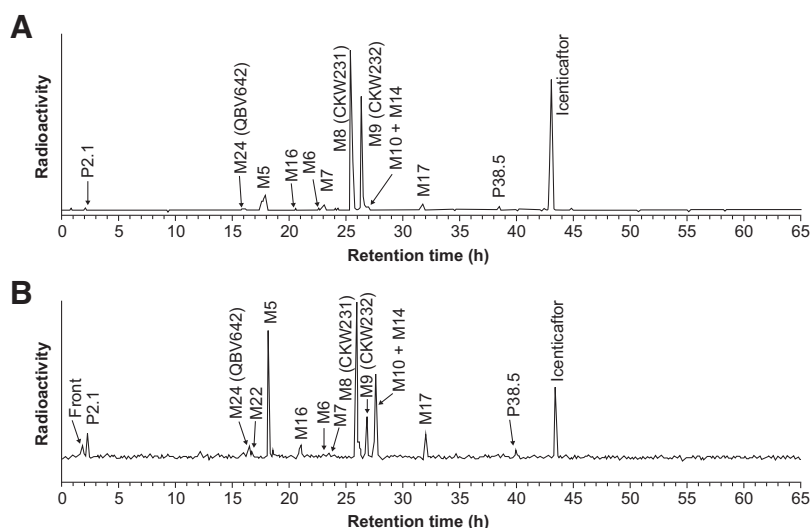


Fig. 3. Representative metabolite profile in plasma at 4 and 48 hours postdose. (A) Plasma metabolite profile at 4 hours (T_{max}). (B) Plasma metabolite profile at 48 hours. The profiles were obtained using HPLC analysis after direct injection of the plasma extract into the HPLC system and subsequent detection of radioactivity in 96-well plates.

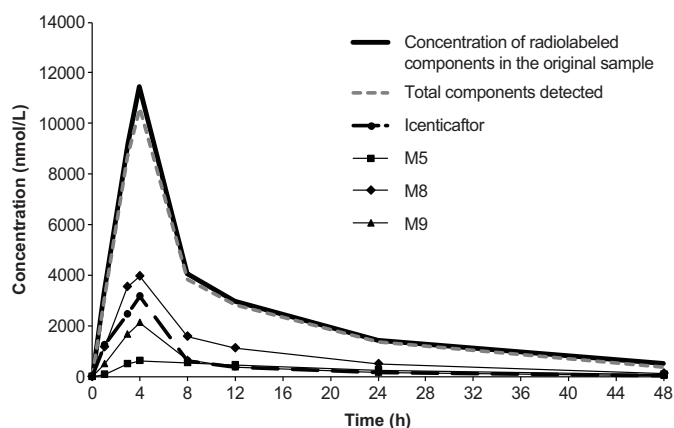


Fig. 4. Concentration-time course of icenticaftor and its main metabolites in plasma at 0–48 hours postdose. Metabolite profiles in plasma were determined from plasma pools of six subjects using HPLC analysis, with radioactivity detection at time points 1, 3, 4, 8, 12, 24, and 48 hours.

Excretion

Total radioactivity was excreted mainly in urine (mean: 92.3% of the total dose) and feces (mean: 5.2% of the total dose); more than 95% of the dose was recovered within 96 hours. By 216 hours postdose, excretion of radioactivity was near complete (97.6%; range: 93.4–99.1%), with less than 3% of the dose being excreted after 96 hours. Figure 8 shows a graph for the cumulative excretion of radioactivity in urine and feces.

Renal excretion of radioactivity was mainly in the form of metabolites (mean \pm S.D.: $92.3 \pm 4.2\%$ of the dose), with minor amounts of unchanged icenticaftor (3.35% of the total dose within 0–96 hours). In feces, unchanged icenticaftor was the most abundant component (2.68% of the dose), with 1.07% excreted as metabolites. The excretion of radioactivity was complete after 9 days (mean: 97.6%; range: 93.4–99.1%).

Overall, the metabolite investigations in excreta indicated that the majority of the dose (80%) was eliminated by direct glucuronidation (M8, M9), with the remaining dose (18%) eliminated by oxidative pathways partly in combination with glucuronidation, mainly M5, and by other metabolic reactions (2%). For the calculation, it was assumed that 100% of the [14 C]icenticaftor dose was recovered and that residual icenticaftor in excreta was formed by hydrolysis of the direct glucuronides in the bladder and intestine.

Systemic clearance (CL_{ss}/F) of [14 C]icenticaftor was moderate (mean \pm S.D.: 56.8 ± 25.6 L/h), and the mean $T_{1/2}$ of total radioactivity in blood and plasma was 15.4 and 20.6 hours, respectively (Table 1).

In Vitro Phenotyping of Icenticaftor Metabolites

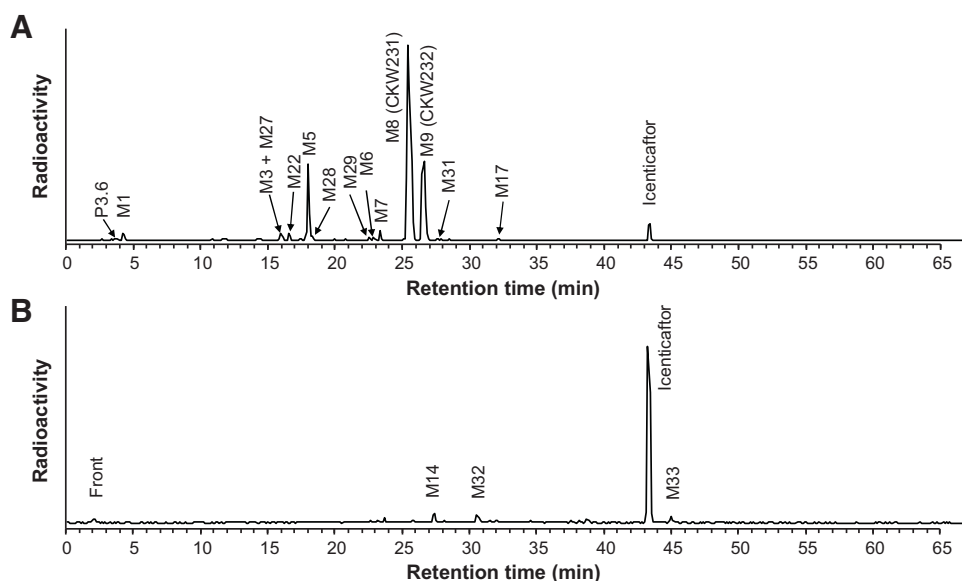
The main metabolic pathways identified in HLMs were mediated by UGT1A9, UGT2B7, CYP1A2, and CYP3A4. Several UGT isoforms (UGT1A3, UGT1A4, UGT1A9, and UGT2B7) were involved in direct glucuronidation of icenticaftor. The formation of the main metabolite M9 (O-glucuronide) was mediated by UGT2B7, with a contribution ratio (fraction of drug systemically available that is converted to metabolites [f_m]) of 13% (Table 3). UGT1A9 was predominantly responsible for the formation of the N-glucuronide (M8), which is a major metabolite of icenticaftor in HLMs. UGT1A3 and UGT1A4 were minor pathways (data not shown). Furthermore, UGT1A7 and UGT1A8 (extrahepatic UGT enzyme isoforms) could catalyze M8 formation (data not shown). The formation of M14, M16, M17, M18, and M19 was mediated by CYP1A2 and CYP3A4, with f_m 11.6% and 19.4%, respectively (Table 3). UGT1A9 had the lowest unbound Michaelis–Menten constant ($K_{m,u}$: $0.34 \mu\text{M}$).

Based on the UGT phenotyping results and the calculated unbound intrinsic clearance values for CYP phenotyping, the contributions of CYPs and UGTs to total icenticaftor metabolism in HLMs (f_m) were estimated as 31% and 69%, respectively (Table 3). Further information on the kinetic parameters and identification of human UGT isoenzymes involved in the glucuronidation of icenticaftor is presented in Supplemental Tables 7 and 8 and representative radiochromatograms are presented in Supplemental Figs. 8 and 9.

Safety

Multiple doses of oral icenticaftor 400 mg and a single oral dose of radiolabeled [14 C]icenticaftor 400 mg were well tolerated in the healthy male subjects. All six subjects received the dosing according to protocol specifications and completed the study. No deaths, serious AEs, or AEs that led to discontinuation were observed. A total of 24 AEs were reported, which were mild in severity (headache, $n = 4$; rhinitis, $n = 3$; nasopharyngitis and somnolence, $n = 2$ each; abdominal discomfort, catheter-site pain, limb injury, pain in extremity, pollakiuria, $n = 1$ each). Eleven events of headache reported in four subjects were considered related to the study drug.

Fig. 5. Representative metabolite profiles in (A) urine and (B) feces. The urine fraction pool (0–96 hours; $N = 6$), containing 90.4% of the total excreted dose, and the feces fraction pool (0–120 hours; $N = 6$) were evaluated using HPLC analysis, with subsequent radioactivity detection in 96-well plates.



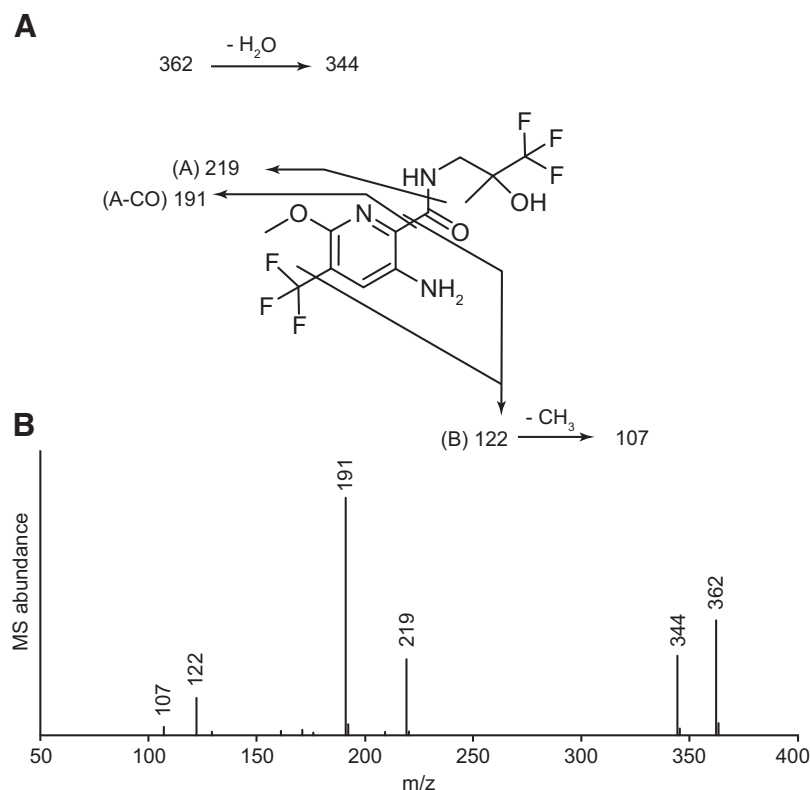


Fig. 6. Electrospray mass spectrum of icenticaftor. (A) Key fragments used for the elucidation of the structure of metabolites. (B) Electrospray ionization, positive ion mode, sample cone voltage of 30 V, trap collision energy ramp of 10–40 eV. The accurate mass measurements were in agreement with the proposed fragmentation. The difference between the calculated and measured masses was ≤ 0.5 mDa for all indicated fragment ions.

Discussion

In a human ADME study, it is assumed that the mean oral absorption can be estimated if the drug and its metabolites are stable against the action of intestinal bacterial enzymes. Initially, the oral absorption of icenticaftor was estimated as 93.4% of the administered dose given that 92.3% of the [14 C]icenticaftor dose was excreted in urine and 1.07%

was excreted in feces as metabolites. Total excretion was largely a result of the renal excretion of icenticaftor as direct glucuronides. In this study, the main glucuronides, M8 and M9, were not detected in feces, but these may have been hydrolyzed by intestinal microorganisms, as described previously for other drugs (Wilson and Nicholson, 2017). Interestingly, M8 and M9 were the main metabolites identified in the bile

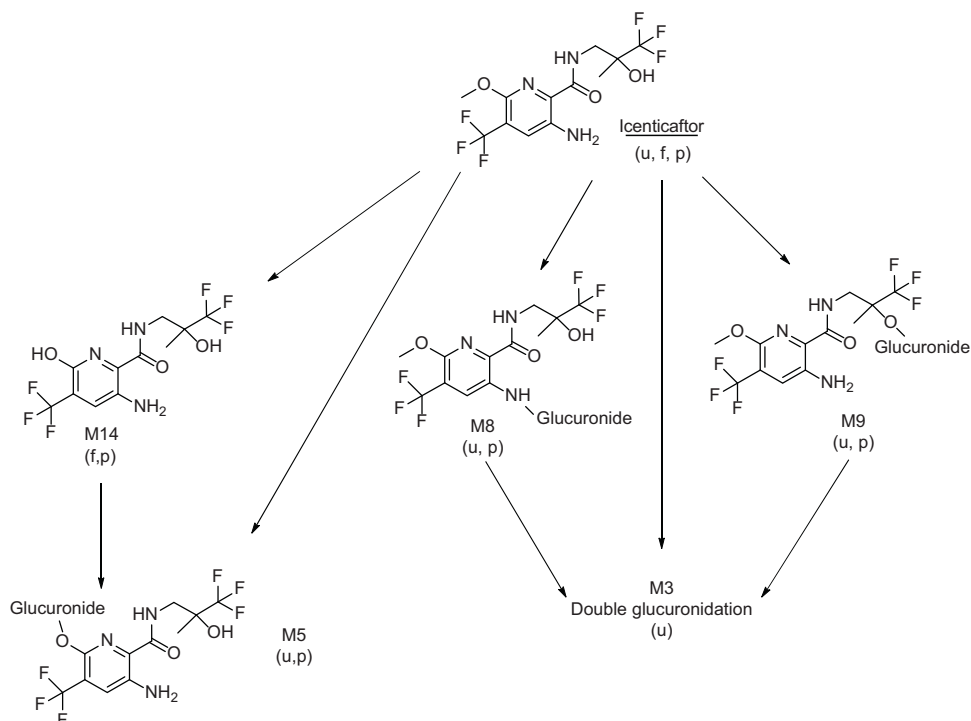


Fig. 7. Scheme of the major biotransformation pathway of icenticaftor in humans (Pathway I). The metabolites were detected in plasma, urine, and/or feces; f, feces; p, plasma; u, urine.

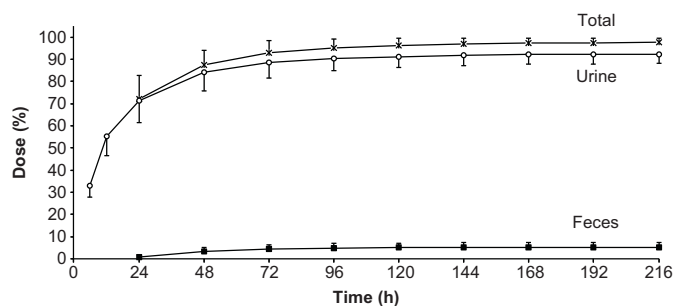


Fig. 8. Cumulative excretion of radioactivity in urine and feces.

(bile duct-cannulated rats), whereas both glucuronides were detected only in trace amounts in the feces of intact rats. This finding suggests that M8 and M9 are not stable against the action of bacterial enzymes in the intestine of rats, a finding that could be extrapolated to humans. Although there are no intravenous data to definitively determine the extent of absorption, given the above findings, absorption could be assumed to be complete for humans (100%), with the major contribution by glucuronidation (80%) largely through renal excretion.

The distribution of icenticaftor within the human body was moderate (V_z/F of 335 L) compared with the volume of water in the body (42 L). Icenticaftor and its metabolites were mainly confined to the plasma compartment, as indicated by the mean blood/plasma AUC_{inf} ratio of 0.579. Therefore, no special affinity of icenticaftor and/or its metabolites for erythrocytes could be concluded.

Peak concentrations of icenticaftor and total radioactivity after oral dosing of 400 mg [^{14}C]icenticaftor to human subjects showed good systemic availability of icenticaftor. In this human ADME study, the steady state AUC (8170 ng*h/ml, Table 1) was identical to the single-dose [^{14}C] AUC_{inf} of icenticaftor (22700 nmol*h/L or 8200 ng*h/ml, Table 2), supporting the validity of the dosing approach. Steady state PK parameters reported for this study were comparable to the results of a previous study of healthy subjects (Kazani et al., 2021). On Day 14 in healthy subjects who received icenticaftor 450 mg twice daily, there was rapid absorption of icenticaftor (T_{max} 4 hours), the mean C_{max} was 2190 ng/ml, and the AUC_{0-12h} was 12100 ng*h/ml (Kazani et al., 2021).

The PK parameters of radioactivity and icenticaftor in plasma and blood had relatively low to moderate variability for C_{max} and AUC, supporting the pooling strategy used for the metabolite pattern investigations. In such cases, time pools across subjects can enable PK calculations for individual metabolites, which may not be detectable in individual subjects, e.g., as for metabolite M5, M14, and M17 (Table 2)

Unchanged icenticaftor and the metabolites M8, M9, and M5 represented the majority of the circulating moieties. Metabolism occurred mainly by N-glucuronidation, O-glucuronidation, and/or O-demethylation and subsequent renal excretion. Approximately 80% of the icenticaftor dose was eliminated by glucuronidation and 18% by glucuronidation and

oxidation, with the remaining 2% elimination occurring by other metabolic reactions.

Interestingly, the data from the in vitro studies conducted with rat and human hepatocytes were comparable and revealed M8 and M9 as major metabolites. However, in the systemic circulation of rats, the metabolites M8 and M9 were only of minor importance when compared with those in circulation in humans. Glucuronides were eliminated mainly through the hepatic biliary route in rats compared with the renal route in humans. This indicates a species difference between rats and humans in the elimination of icenticaftor and emphasizes the importance of conducting a human ADME study.

Phenotyping analysis suggested M8 formation through UGT1A9 is likely a major component in icenticaftor metabolism in HLMs ($f_m = 56\%$), followed by CYP enzymes (CYP1A2 with partial contribution of CYP3A4; $f_m = 31\%$) and UGT2B7 (M9 formation, $f_m = 13\%$). The fractional contributions calculated using the in vitro data support the in vivo data, where UGT and CYP f_m values of 80–98% and 0.34–18%, respectively, were determined.

In terms of safety, simple O-glucuronides, O-sulfates, and quaternary N-glucuronides can be considered benign from a human safety perspective, but adequate animal exposure may be required for those that undergo chemical rearrangement (e.g., acyl glucuronides) because of reactivity concerns (Luffer-Atlas and Atrakchi, 2017; US Food and Drug Administration, 2020). Notably, the main metabolites of icenticaftor (M8 and M9) were simple O- or N-glucuronides, not acyl glucuronides; therefore, safety concerns were not expected. Additionally, guidelines state that assessment of direct N- or O-glucuronides (M8 and M9, respectively) may not be needed because Phase 2 conjugation reactions generally render a compound more water soluble, thereby eliminating the need for further evaluation (European Medicines Agency, 2012c; US Food and Drug Administration, 2020). Despite this, M8 and M9 metabolites were evaluated in toxicity studies because of the abundance of both metabolites compared with the abundance of the parent drug.

Human ADME studies are typically performed as single-dose studies (Ramamoorthy et al., 2022), which are considered sufficient if there is no dose or time dependency in the first-pass metabolism or elimination of the drug (European Medicines Agency, 2012b). The administration of a radioactive dose of icenticaftor at steady state was deemed necessary to further investigate the rate and route of elimination of icenticaftor following the observation of dose nonlinearity after the administration of a single dose of icenticaftor in a previous investigation. If there is dose- or time-dependent elimination of a drug, then the contribution of different pathways may be different at steady state than at single-dose conditions. Drug accumulation is one of the factors influencing drug dose nonlinearity; however, this may also occur because of other reasons, including saturation of enzymes (Pharmacy180.com). The results would likely have been different if the hADME study had been performed in a single-dose study because different fractions are expected to be metabolized per

TABLE 3
Summary of in vitro CYP and UGT phenotyping results of icenticaftor

Enzyme	Metabolite	$K_{m,u}$ (μM) ^a	V_{max} (pmol/min/mg) ^b	$CL_{int,u}$ (μl /min/mg)	f_m (%)
CYP1A2	M14, M16, M17, M18, M19	4.61	14.5	3.14	11.6
CYP3A4	M14, M16, M17, M18, M19	13.0	68.1	5.24	19.4
UGT1A9 ^c	M8	0.34	5.32	15.2	56.0
UGT2B7	M9	4.64	17.0	3.67	13.0

$CL_{int,u}$, unbound intrinsic clearance; V_{max} , maximum velocity (reaction velocity at saturating substrate concentration).

^a Base fraction of the unbound test substance in microsomes = $0.2571 \times (\text{mg of microsomal protein})^2 - 0.5798 \times (\text{mg of microsomal protein}) + 0.7995$ (see Supplemental Material [Section 8], Supplemental Table 8, and Supplemental Fig. 10).

^b Based on liver enzyme abundance of 52.0, 137, 31, and 71 pmol/mg for CYP1A2, CYP3A4, UGT1A9, and UGT2B7, respectively.

^c UGT1A3 and UGT1A4 were also identified as minor metabolizing enzymes.

pathway due to the saturation of at least UGT1A9 ($K_{m,u}$: 0.34 μM). This is indicated by a more than 2-fold higher AUC and a slightly higher C_{max} at steady state than that after a single-dose icenticaftor. The nonlinear PK observed after the administration of both single and multiple doses of icenticaftor is most likely due to saturation of UGT1A9 in the intestine and liver and consequent CL/F decreases with increasing plasma concentrations. This conclusion is supported by the low $K_{m,u}$ value (0.34 μM) of UGT1A9 in the major metabolic pathway.

The present study was tailored to suit the PK characteristics of icenticaftor, in agreement with the guidelines set by applicable health authorities, namely Appendix V of the European Medicines Agency “Guideline on the Investigation of Drug Interactions” and the US Food and Drug Administration draft guidance on the design of human mass balance studies for identifying and quantifying the main elimination pathways in vivo (European Medicines Agency, 2012b; US Food and Drug Administration, 2024). Administering a single dose of radiolabeled icenticaftor when steady-state concentrations have been reached with the nonradiolabeled drug can reveal the metabolic and elimination fate of the radiolabeled drug. While there may be concerns that such studies would not provide information about the saturating behavior of the radiolabeled drug or the metabolic profile of the metabolites at steady state, the European Medicines Agency states that there is no need to estimate AUC_{0-T} (area under the curve [AUC] limited to the end of a dosing interval) because the $\text{AUC}_{0-\text{inf}}$ of metabolites observed at steady state will reflect the AUC_{0-T} of the metabolite (European Medicines Agency, 2013). Hence, this study supported the development of icenticaftor by providing invaluable insights into its disposition and its low victim drug–drug interaction potential.

Conclusions

Icenticaftor was extensively metabolized, with both the original compound and metabolites confined mainly to the plasma compartment, where the metabolites M8, M9, and M5 represented the main metabolites in circulation. Icenticaftor was eliminated mainly through metabolism and subsequent renal excretion (92.3% excretion through urine [kidneys] and 5.25% through feces [liver/bile]).

Acknowledgments

Financial support for medical editorial assistance was provided by Novartis Pharma AG, Basel, Switzerland. The authors thank Sorcha McGinty; Helen Swainston (Novartis Global Business Solutions, Dublin, Ireland); and Rina Vekaria Passmore (Novartis Global Business Solutions, London, UK) for their medical editorial assistance with this manuscript.

Data Availability

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Material.

Authorship Contributions

Participated in research design: Glaenzel, Huth, Hackling.

Conducted experiments: Glaenzel, Leuthold, Meissner.

Contributed new reagents or analytic tools: Glaenzel, Eggimann; Meissner.

Performed data analysis: Glaenzel, Huth, Hackling, Leuthold, Meissner, Bebrevska.

Wrote or contributed to the writing of the manuscript: Glaenzel, Huth, Eggimann, Hackling, Leuthold, Meissner, Bebrevska.

References

- Coppola P, Andersson A, and Cole S (2019) The Importance of the Human Mass Balance Study in Regulatory Submissions. *CPT Pharmacometrics Syst Pharmacol* 8:792–804.
- European Medicines Agency (2012a) Kalydeco summary of product characteristics. https://www.ema.europa.eu/en/documents/product-information/kalydeco-epar-product-information_en.pdf. Accessed August 01, 2023.
- European Medicines Agency (2012b) Guideline on the investigation of drug interactions CPMP/EWP/560/95/Rev. 1 Corr. 2. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-investigation-drug-interactions-revision-1_en.pdf. Accessed July 20, 2023.
- European Medicines Agency (2012c) ICH guideline M3 (R2) - questions and answers. https://www.ema.europa.eu/en/documents/other/international-conference-harmonisation-technical-requirements-registration-pharmaceuticals-human-use_en.pdf. Accessed July 31, 2023.
- European Medicines Agency (2013) Overview of comments received on ‘Guideline on the Investigation of Drug Interactions’ (EMA/CHMP/EWP/125211/2010). https://www.ema.europa.eu/en/documents/other/overview-comments-received-guideline-investigation-drug-interactions_en.pdf. Accessed July 20, 2023.
- Grand DL, Gosling M, Baettig U, Baha P, Bala K, Brocklehurst C, Budd E, Butler R, Cheung AK, Choudhury H, et al. (2021) Discovery of Icenticaftor (QBW251), a Cystic Fibrosis Transmembrane Conductance Regulator Potentiator with Clinical Efficacy in Cystic Fibrosis and Chronic Obstructive Pulmonary Disease. *J Med Chem* 64:7241–7260.
- Hanssens LS, Duchateau J, and Casimir GJ (2021) CFTR Protein: Not Just a Chloride Channel? *Cells* 10:2844.
- Kazani S, Rowlands DJ, Bottoli I, Milojevic J, Alcantara J, Jones I, Kulmatycki K, Machineni S, Mostovyy L, Nicholls I, et al. (2021) Safety and efficacy of the cystic fibrosis transmembrane conductance regulator potentiator icenticaftor (QBW251). *J Cyst Fibros* 20:250–256.
- Lindmark B, Li X-Q, Bhattacharya C, Housler G, Heijer M, Bragg RA, Gränfors M, Pelay-Gimeno M, Vaes WHJ, Menakuru S, et al. (2023) Mass Balance and Absorption, Distribution, Metabolism, and Excretion Properties of Balcinrenone following Oral Administration in Combination with Intravenous Microtracer in Healthy Subjects. *Drug Metab Dispos* 51:995–1004.
- Luffer-Atlas D and Atrachchi A (2017) A decade of drug metabolite safety testing: industry and regulatory shared learning. *Expert Opin Drug Metab Toxicol* 13:897–900.
- Mall MA, Criner GJ, Miravittles M, Rowe SM, Vogelmeier CF, Rowlands DJ, Schoenberger M, and Altman P (2023) Cystic fibrosis transmembrane conductance regulator in COPD: a role in respiratory epithelium and beyond. *Eur Respir J* 61:2201307.
- Martinez FJ, Criner GJ, Gessner C, Jandl M, Scherbovsky F, Shinkai M, Siler TM, Vogelmeier CF, Voves R, Wedzicha JA, et al. (2023) Icenticaftor, a CFTR Potentiator, in COPD: a Multi-center, Parallel-Group, Double-Blind Clinical Trial. *Am J Respir Crit Care Med* 208:417–427.
- Moss RB, Flume PA, Elborn JS, Cooke J, Rowe SM, McColley SA, Rubenstein RC, and Higgins M, VX11-770-110 (KONDUCT) Study Group. (2015) Efficacy and safety of ivacaftor in patients with cystic fibrosis who have an Arg117His-CFTR mutation: a double-blind, randomised controlled trial. *Lancet Respir Med* 3:524–533.
- Pharmacy180.com Biopharmaceutics and Pharmacokinetics: Nonlinear Pharmacokinetics. Causes of. <https://www.pharmacy180.com/article/causes-of-nonlinearity-2529/>. Accessed August 13, 2024.
- Ramamoorthy A, Bende G, Chow ECY, Dimova H, Hartman N, Jean D, Pahwa S, Ren Y, Shukla C, Yang Y, et al. (2022) Human radiolabeled mass balance studies supporting the FDA approval of new drugs. *Clin Transl Sci* 15:2567–2575.
- Ramsey BW, Davies J, McElvaney NG, Tullis E, Bell SC, Dřevínek P, Griese M, McKone EF, Wainwright CE, Konstan MW, et al. and Grp VS. (2011) A CFTR potentiator in patients with cystic fibrosis and the G551D mutation. *N Engl J Med* 365:1663–1672.
- Rowe SM, Heltshe SL, Gonska T, Donaldson SH, Borowitz D, Gelfond D, Sagel SD, Khan U, Mayer-Hamblett N, Van Dalfsen JM, et al. and Fibrosis GIC. (2014) Clinical mechanism of the cystic fibrosis transmembrane conductance regulator potentiator ivacaftor in G551D-mediated cystic fibrosis. *Am J Respir Crit Care Med* 190:175–184.
- Rowe SM, Jones I, Dransfield MT, Haque N, Gleason S, Hayes KA, Kulmatycki K, Yates DP, Danahy H, Gosling M, et al. (2020) Efficacy and Safety of the CFTR Potentiator Icenticaftor (QBW251) in COPD: Results from a Phase 2 Randomized Trial. *Int J Chron Obstruct Pulmon Dis* 15:2399–2409.
- Shi J, Li H, Yuan C, Luo M, Wei J, and Liu X (2018) Cigarette Smoke-Induced Acquired Dysfunction of Cystic Fibrosis Transmembrane Conductance Regulator in the Pathogenesis of Chronic Obstructive Pulmonary Disease. *Oxid Med Cell Longev* 2018:6567578.
- US Food and Drug Administration (2012) Kalydeco prescribing information. https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/203188s0221_207925s0031bl.pdf. Accessed August 01, 2023.
- US Food and Drug Administration (2020) Safety testing of drug metabolites: guidance for industry. <https://www.fda.gov/media/72279/download>. Accessed August 08, 2023.
- US Food and Drug Administration (2024) Clinical pharmacology considerations for human radiolabeled mass balance studies: Guidance for industry. <https://www.fda.gov/media/158178/download>. Accessed August 16, 2024.
- Van Goor F, Hadida S, Grootenhuys PDJ, Burton B, Cao D, Neuberger T, Turnbull A, Singh A, Joubbran J, Hazlewood A, et al. (2009) Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. *Proc Natl Acad Sci U S A* 106:18825–18830.
- Wilson ID and Nicholson JK (2017) Gut microbiome interactions with drug metabolism, efficacy, and toxicity. *Transl Res* 179:204–222.

Address correspondence to: Ulrike Glaenzel, PK-Sciences, Novartis Pharma AG, Fabrikstrasse 14, WSJ-153.1.02 Basel 4056, Switzerland. E-mail: ulrike.glaenzel@novartis.com

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

Ulrike Glaenzel, Felix Huth, Fabian Eggimann, Melissa Hackling, Luc Alexis Leuthold, Axel Meissner, and Lidiya Bebrevska

Novartis Pharma AG, Basel, Switzerland (U.G., F.H., F.E., L.A.L., A.M., L.B.); Novartis Pharmaceuticals Corporation, East Hanover, New Jersey, USA (M.H.)

Table of contents

1. Inclusion and Exclusion Criteria.....	2
2. Sample Collection and Preparation	3
3. Analysis of Total Radioactivity.....	5
4. Determination of Plasma Icenticaftor Concentrations	7
5. Determination of Metabolite Profiles in Plasma, Urine, and Feces.....	8
6. Synthesis of Reference Standards	11
7. In Vitro Phenotyping of Cytochrome P450 (CYP) Enzymes.....	13
8. In Vitro Phenotyping of Uridine Diphosphate Glucuronosyltransferase (UGT) Isozymes ..	17
9. In Vitro Biotransformation in Mouse, Rat, Monkey, and Human Hepatocytes	20
10. Rat ADME Study	22
11. References.....	31
12. Supplemental Tables.....	32
13. Supplemental Figures	50

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/m²; 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 [¹⁴C]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 [¹⁴C]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 10°C (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

[¹⁴C] 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 [¹⁴C] 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 μ L of internal standard ($[^{13}\text{CD}_3]$ icenticaftor) to 100 μ L of aliquots of the collected plasma samples, followed by protein precipitation with acetonitrile and centrifugation. Following this, 2- μ L 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 \times 4.6 mm, 2.6 μ m 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 \rightarrow m/z 191.0 (100 ms dwell time); for $[^{13}\text{CD}_3]$ icenticaftor, m/z 366.0 \rightarrow 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 ($18,000 \times g$, 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 $18,000 \times g$. 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 $12,000 \times g$ 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 $12,000 \times g$ 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 ¹⁴C-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 [¹⁴C]licenticaftor 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 [¹⁴C]licenticaftor 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 MgCl₂ (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 Liebig™ 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 [¹⁴C]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 [¹⁴C]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 [¹⁴C]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 μ M and 8 μ M [14 C]icenticaftor.

Inhibition of [14 C]icenticaftor Biotransformation by Chemical Inhibitors

The oxidative metabolism of 1 μ M [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 μ M furafylline (CYP1A2 inhibitor), 0.0391, 0.0781, 0.156, 0.313, 0.625, 1.25, 2.5, 5, and 10 μ M ticlopidine (CYP2B6/CYP2C19 inhibitor), 0.00781, 0.0156, 0.0313, 0.0625, 0.125, 0.25, 0.5, 1, and 2 μ M montelukast (CYP2C8 inhibitor), 0.0781, 0.156, 0.313, 0.625, 1.25, 2.5, and 5 μ M sulfaphenazole (CYP2C9 inhibitor), 0.00391, 0.008, 0.0156, 0.0313, 0.0625, 0.125, 0.250, 0.50, 1, and 2 μ M loratadine (CYP2C19 inhibitor), 0.00781, 0.0156, 0.0313, 0.0625, 0.125, 0.25, 0.5, 1, and 2 μ M quinidine (CYP2D6 inhibitor), 1.56, 3.125, 6.25, 12.5, 25, 50, and 100 μ M sodium diethyldithiocarbamate (DETC; CYP2E1 inhibitor), 0.00781, 0.0156, 0.0313, 0.0625, 0.125, 0.25, 0.5, and 1 μ M ketoconazole (CYP3A4/5 inhibitor), 0.0391, 0.0781, 0.156, 0.313, 0.625, 1.25, 2.5 and 5 μ M azamulin (CYP3A4/5 inhibitor), and 2.5, 5, 10, 20, 40, 80, and 160 μ M 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 μ m, 5 \times 2.1 mm (Waters, Milford, MA, USA)

Pre-column	Aquity UPLC HSS T3; 1.8 μ m, 150 \times 2.1 mm (Waters, Milford, MA, USA)		
Temperature	60°C (HPLC gradient [A]); 40°C (HPLC gradient [B])		
Injection	900 μ L sample loop		
Mobile phase	A: ammonium carbamate 10 mM, pH 7.5 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 [¹⁴C]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 [¹⁴C]icenticaftor were prepared in acetonitrile. HLMS (stock solution 20 mg protein/mL) or recombinant UGTs were pre-incubated with 50- or 200- μ g 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 mM Tris buffer (pH 7.5, at 37°C). Typical incubations of 400 μ L were prepared as follows: 20 μ L of 100 mM MgCl₂ (5 mM final concentration), substrate (1 μ M and 10 μ M), and pre-incubated proteins (15 μ L of alamethicin stock solution mixed with 750 μ L 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 Liebig 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 [¹⁴C]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 [¹⁴C]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

[¹⁴C]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:

$$f_{u,mic} = 0.2571 \times [\text{mg of microsomal protein}]^2 - 0.5798 \times (\text{mg of microsomal protein}) + 0.7995,$$

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 [¹⁴C]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 [¹⁴C]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 μ m, 5 \times 2.1 mm (Waters, Milford, MA, USA)		
Temperature	60°C		
Injection	900 μ L sample loop		
Mobile phase	A: ammonium carbamate 10 mM, pH 7.5 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 μ M concentrations of [14 C]icenticaftor. A 1.0 mM stock solution of [14 C]icenticaftor in DMSO was used for the 1 μ M incubation and a 13.7 mM stock solution of [14 C]icenticaftor in DMSO was used for the 10 μ M and 30 μ M 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 $\sim 1 \times 10^6$ viable cells/mL after viability determination. The incubations were started by addition of [^{14}C]jenticaftor as a 13.7 mM solution in DMSO (2.2 $\mu\text{L}/\text{mL}$ hepatocytes) for the 30 μM incubations, (0.7 $\mu\text{L}/\text{mL}$ hepatocytes) for the 10 μM incubation and as a 1.0 mM solution in DMSO (1.0 $\mu\text{L}/\text{mL}$ hepatocytes) for the 1 μM incubation. The initial concentrations of [^{14}C]jenticaftor 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 $\times g$, 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 ¹⁴C-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 [¹⁴C]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 Long Evans rats (crt:(LE)BR), pigmented, males
Supplier	Han:WIST: Harlan, The Netherlands 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.

^aFour 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

[¹⁴C]lenticaftor 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]lenticaftor (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	4.62	0.329	2.71 ^b	1.52	6.69	4.41
excretion						
(IV)						

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

^a0.5% (w/v) methyl cellulose containing 0.5% (v/v) Tween 80.

^b5.0% (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 $36,670 \times g$ 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 $\times g$ 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 $\times g$ 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 $\times g$ 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 $\times g$ 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 $\times g$ 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 μ m, 5 \times 2.1 mm	
Analytical column	Waters HSS T3 (Milford, MA, USA) 1.8 μ m, 150 \times 2.1 mm	
Temperature of guard and analytical column	40°C	
Injection volume	10–50 μ L injected through a 100 μ L sample loop	
Mobile phase	A: Ammonium carbamate 10 mM, pH 7.5 B: Acetonitrile For deuterium exchange experiments, ammonium carbamate (10 mM, pH 7.5) in deuterium oxide was used	
Flow rate	500 μ L/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	

<p>Online radioactivity detection</p>	<p>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).</p>
<p>Offline radioactivity detection</p>	<p>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</p>

	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 Cone voltage: 30 V Nebulizer gas: nitrogen (7 bar) Cone gas: nitrogen (25 L/h) Desolvation gas: nitrogen (800 L/h) Source block temperature: 120°C Desolvation temperature: 200°C
Collisional activation	Gas: argon, (7.6×10^{-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 [¹⁴C]icenticaftor at steady state.

Component (in order of elution)	Sample collection time (h)							AUC _{0-48h} ^a	
	1	3	4	8	12	24	48	nM*h	% ^b
	Concentration nM								
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.

^aCalculated using the linear trapezoidal method; concentrations at time zero taken as zero.

^bPercentage of total radiolabeled components in the original sample.

^cPercent 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.

^dPercent 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.

^eSum of the residual radioactive peaks. (All individual peaks have $AUC_{0-48h} \leq 0.14\%$.)

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

Component	Excretion (% of dose)		
	Urine	Feces	Total
	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.

^aSum of residual radioactive peaks. No single component had a contribution of $\geq 0.23\%$ of the dose.

^bSum 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.

^aTrace 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 (<i>m/z</i>)					
Component	Fragments				Additional major signals
	[M+H] ⁺	A [*]	A-CO [*]	B [*]	
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 ₂ O] ⁺ , 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 ₂ O] ⁺ , 376, 358, 314, 297, 278, 236 ^a , 233, 141, 95
M5	524	205 ^a	177 ^a		348 ^a , 330 [M+H – H ₂ O – C ₆ H ₈ O ₆] ⁺ , 122
M28	428				236, 234 [M+H – H ₂ O – C ₆ H ₈ O ₆] ⁺ , 219 ^{a,b} , 191 ^{a,b} , 122 ^{a,b} , 206, 148
M16	354				336 [M+H – H ₂ O] ⁺ , 318, 218, 193, 165, 97
M29	554				536 [M+H – H ₂ O] ⁺ , 360 [M+H – H ₂ O – C ₆ H ₈ O ₆] ⁺ , 297, 261, 233, 219 ^{a,b} , 191 ^{a,b} , 122 ^{a,b}
M6	554				536 [M+H – H ₂ O] ⁺ , 518, 500, 456, 438, 420, 402, 378 ^a , 360 [M+H – H ₂ O – C ₆ H ₈ O 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 ₆ H ₈ O ₆] ⁺ , 344, 208, 129

M8	538	219 ^a	191 ^a	122 ^a	520[M+H – H ₂ O] ⁺ , 502, 484, 440, 404, 362 ^a , 344 [M+H – H ₂ O – C ₆ H ₈ O ₆] ⁺ , 297
M9	538	219 ^a	191 ^a	122 ^a	520 [M+H – H ₂ O] ⁺ , 502, 484, 440, 362 ^a , 344 [M+H– H ₂ O – C ₆ H ₈ O ₆] ⁺
M14	348	205	177		330 [M+H – H ₂ O] ⁺ , 310 [M+H – H ₂ O – HF] ⁺ , 149, 122
M31	554				536 [M+H – H ₂ O] ⁺ , 378 ^a , 360 [M+H – H ₂ O – C ₆ H ₈ O ₆] ⁺ , 342, 237, 219 ^{a,b} , 191 ^{a,b} , 142, 122 ^{a,b}
M10	378	235	207		360 [M+H – H ₂ O] ⁺ , 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 ₂ O] ⁺
M17	236	219	191	122	107
Icenticaftor	362	219	191	122	344 [M+H – H ₂ O] ⁺ , 107
M33	404	219 ^d	191 ^d	122 ^d	386 [M+H – H ₂ O] ⁺ , 362 [M+H – C ₂ H ₂ O] ⁺ , 344, 261, 233, 144

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

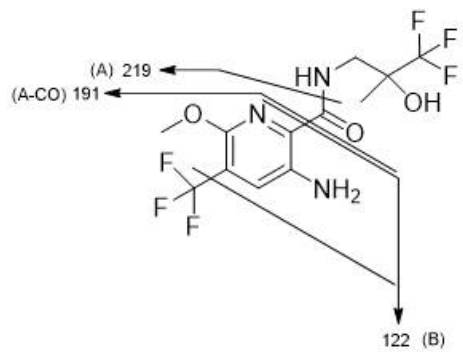
^aThe fragment was likely formed after neutral loss of anhydroglucuronic acid (C₆H₈O₆).

^bThe fragment was likely formed after loss of oxygen (O).

^cThe fragment was likely formed after loss of the SO₃ moiety.

^dThe 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.

Component	Proportion of radioactivity (% of total)			
	Species			
	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.

^aNo single additional component had >1.00% of total radioactivity.

Radiolabeled components following incubation of 10 µM [¹⁴C]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	Feces	Bile	Total
	0–48 hours	0–48 hours	0–48 hours	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
<hr/>				
Sum of additional components	1.75	0.141	4.05	5.94
<hr/>				
Total detected ^b	26.3	2.80	59.1	88.2
<hr/>				
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.

^aPeaks were identified by retention time only.

^bTotal 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 [¹⁴C]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.

Component	Excretion (% of dose)		
	Urine 0–96 h	Feces 0–96 h	Total 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.

^aPeaks 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 [¹⁴C]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 \pm SD)	V_{max} ($\text{pmol}/\text{min}/\text{mg}$) (mean \pm SD)	CL_{int} ($\mu\text{L}/\text{min}/\text{mg}$) (mean)	Relative contribution (%) (mean)
M8	10.3 \pm 2.7	89.3 \pm 8.0	8.67	76.1
M9	7.18 \pm 2.3	15.0 \pm 1.5	2.09	18.3
Total	8.88 \pm 1.7	101 \pm 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 \pm SD)	K_m (μM) ^b	V_{max} ($\mu\text{mol}/\text{min}/\text{mg}$) (mean \pm SD)	$CL_{\text{int,u}}$ ($\mu\text{L}/\text{min}/\text{mg}$) (mean)
CYP1A2	7.43 \pm 1.4	4.59	401 \pm 41.4	87.4
CYP3A4	19.4 \pm 2.1	13.5	717 \pm 59	53.1
UGT1A8	277 \pm 20	159	131 \pm 17	0.824
UGT1A9	0.593 \pm 0.071	0.34	41.0 \pm 1.1	121
UGT2B7 ^a	8.09 \pm 0.86	4.64	7.44 \pm 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).

^aM9 formation rate. For other UGTs, the total metabolite formation rate was included in the kinetic analysis.

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

B. Correlation analysis.

Marker reaction	Enzyme	Linear regression coefficient (R)		
		M8	M9	Sum of all metabolites
17 β -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
17 β -Estradiol 17 β -glucuronidation	Unknown UGT	-0.185	0.607	0.053

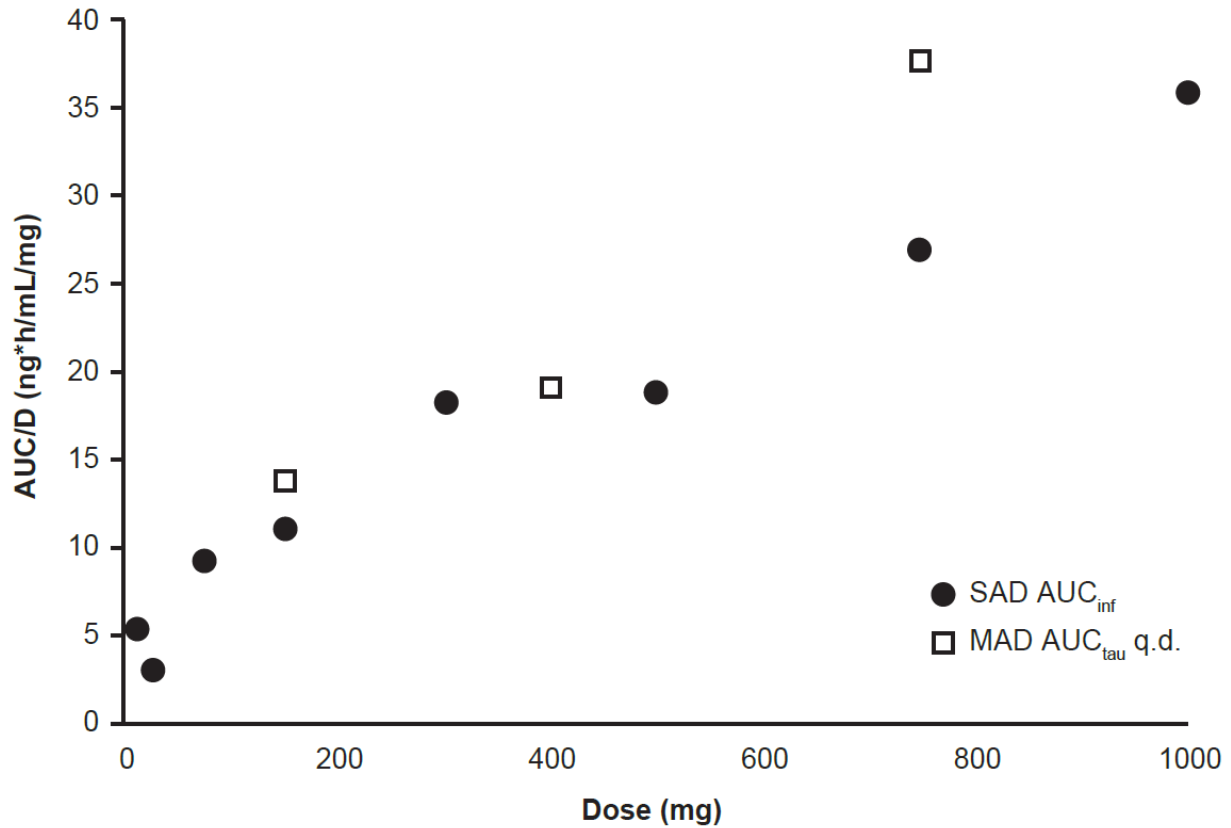
HLM, human liver microsome; UGT, uridine diphosphate glucuronosyltransferase.

HLMs (n = 16) were incubated with 3 μ M [¹⁴C]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.



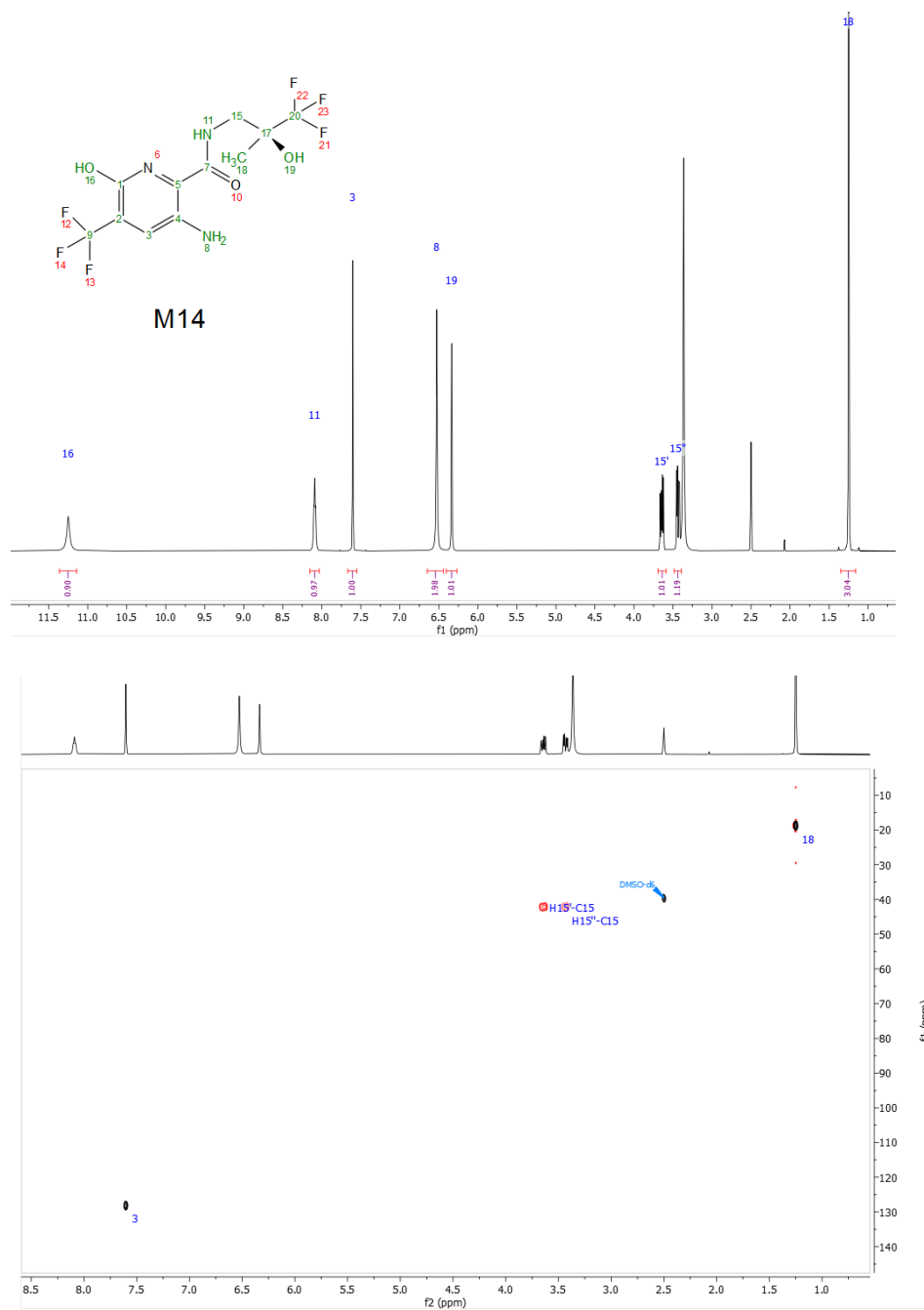
Supplemental Fig. 2. Study design.

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

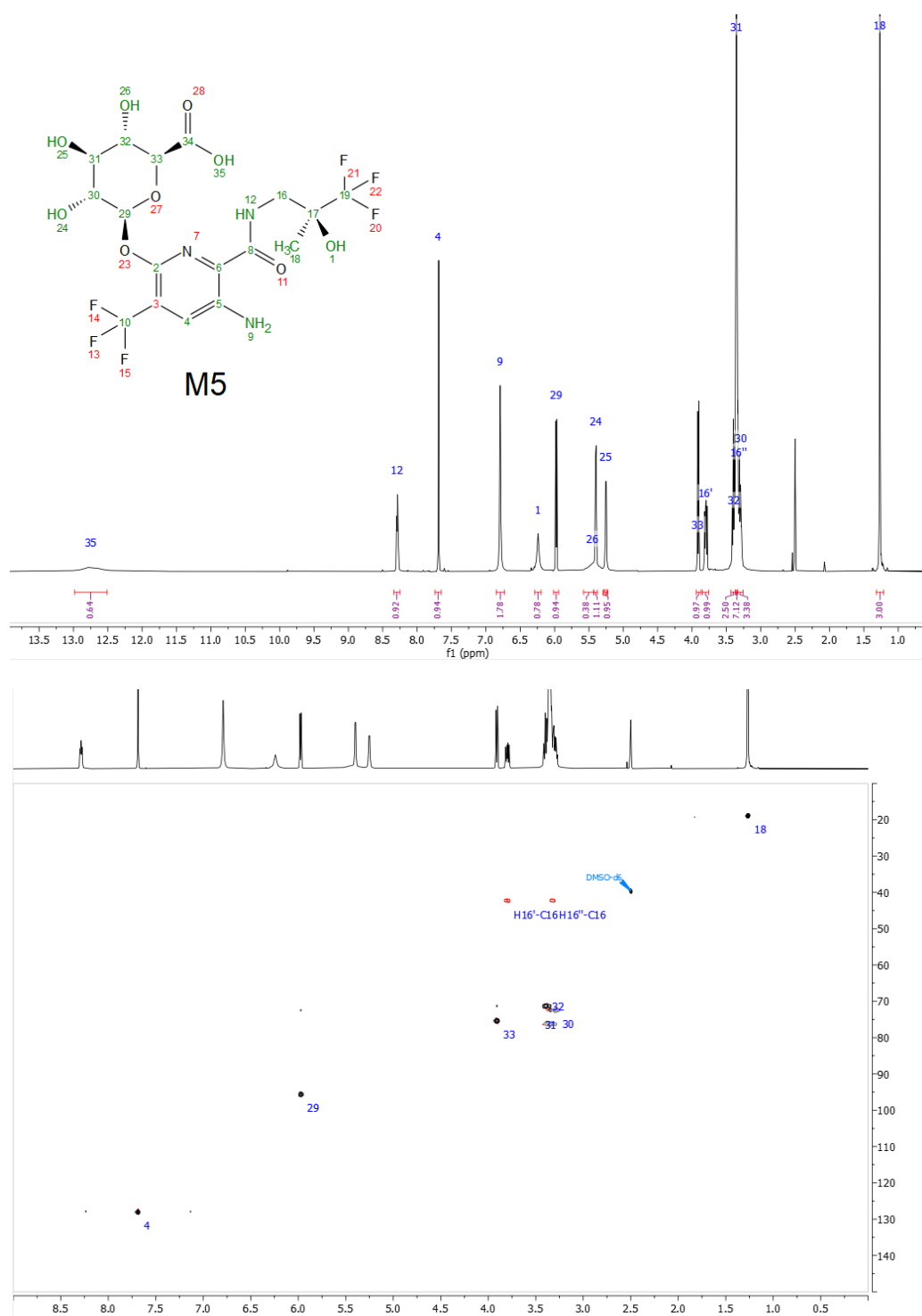
Screening						Treatment												Observational F/U						Safety F/U				
	AM	Icenticaftr 400 mg				[¹⁴ C]Icenticaftr 400 mg				Icenticaftr 400 mg																		
-28 to -1	Day	1	2	3	4	5				6	7	8	9	10	11	12	13	14	15	16	17	18	20	23	26	32	33	
	PM	Icenticaftr 400 mg				Icenticaftr 400 mg				Icenticaftr 400 mg																	EOS	
	Confined to site	X														X	X	X	X									

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).

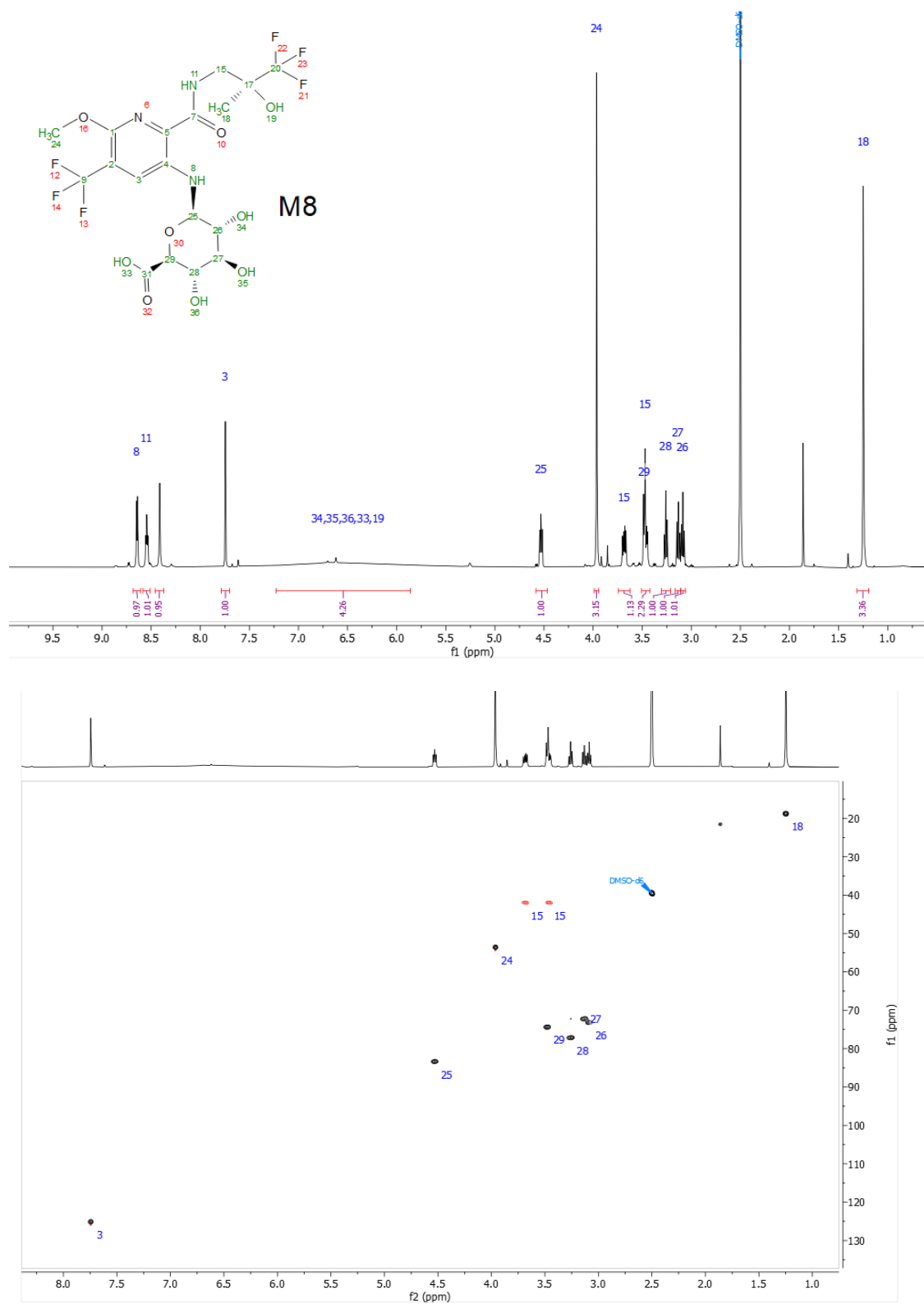
A



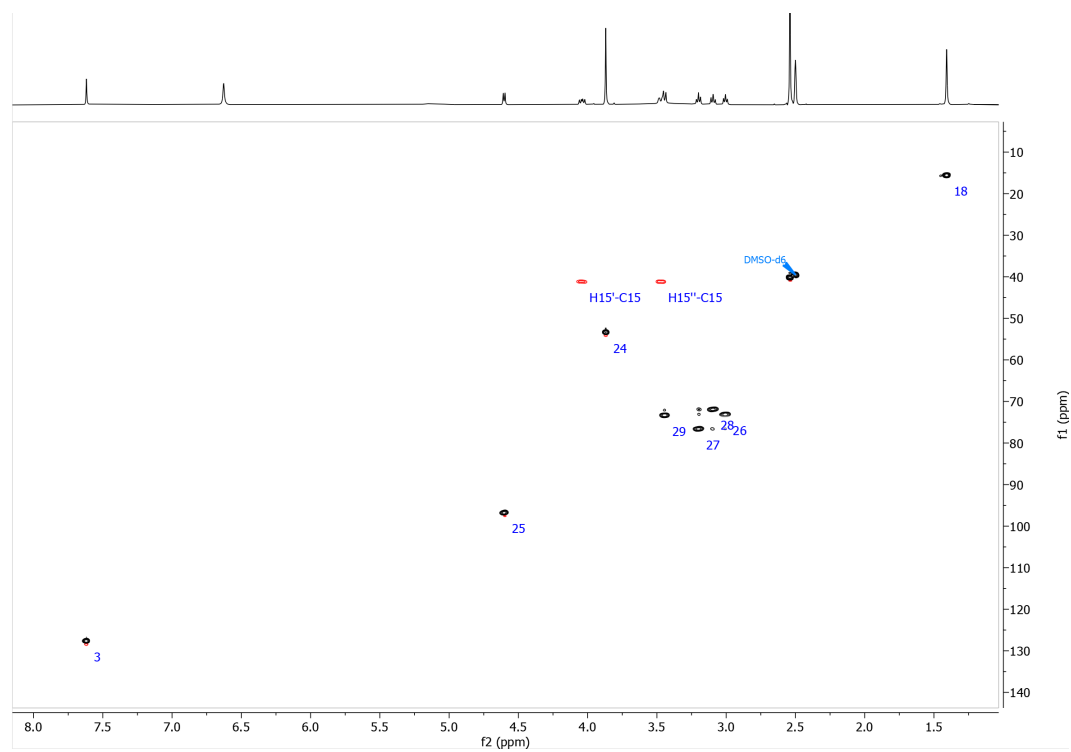
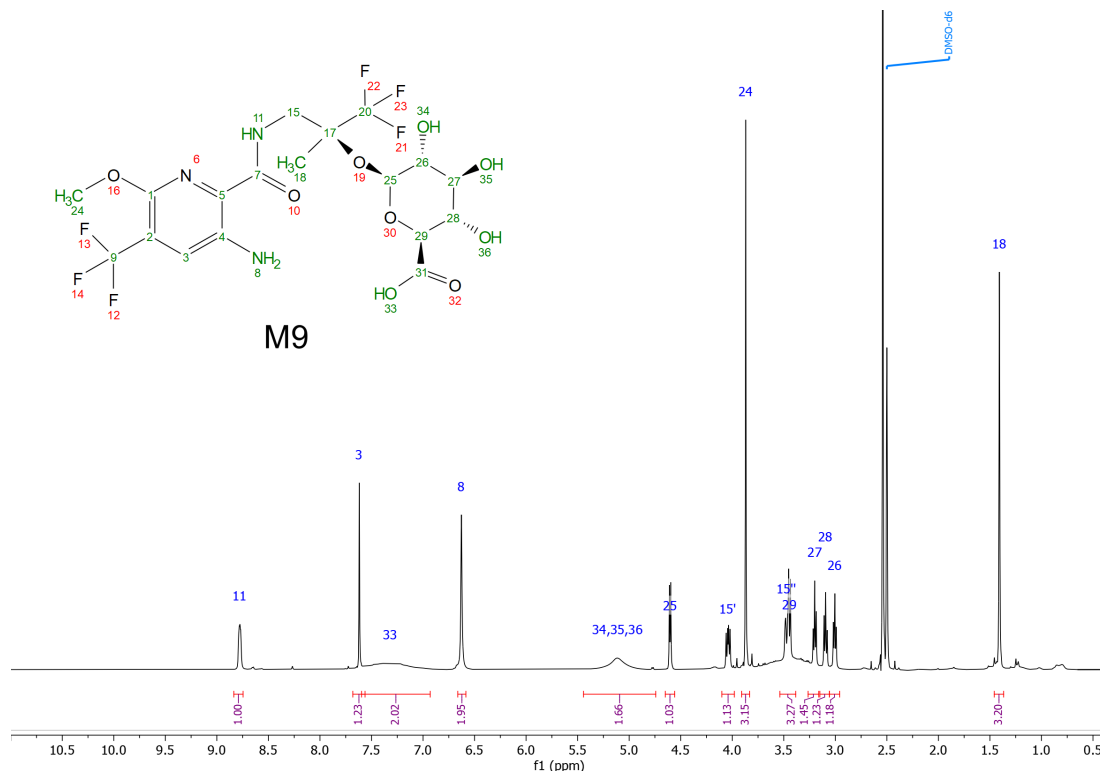
B



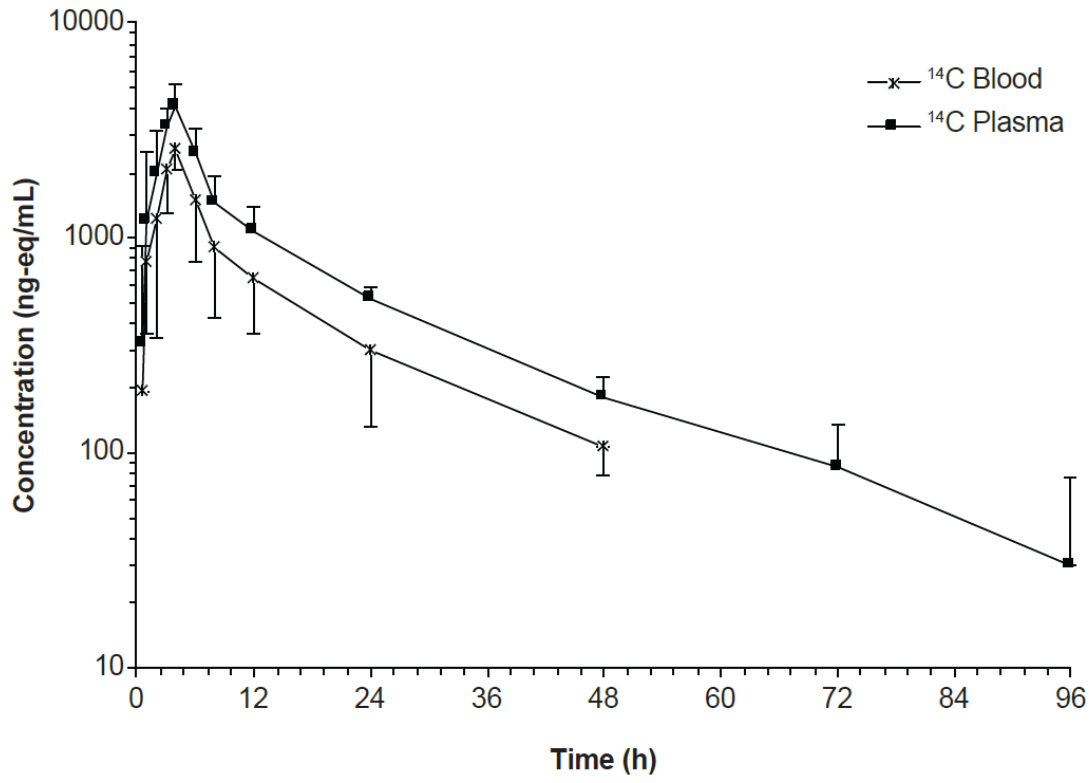
C



D



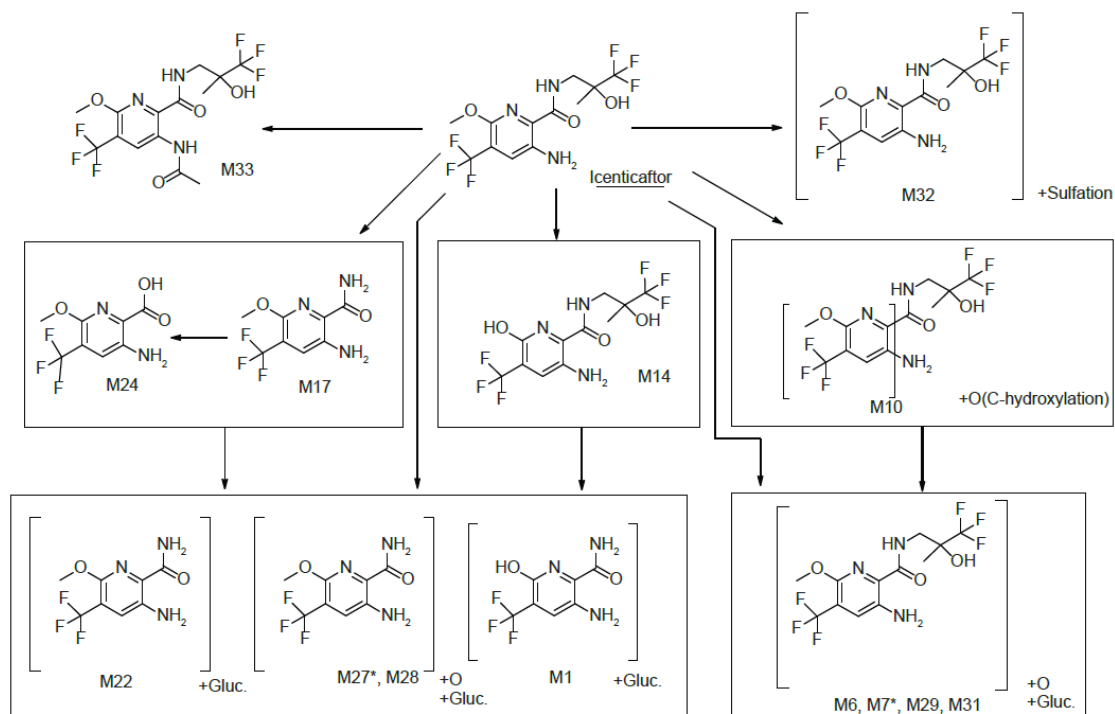
Supplemental Fig. 4. Total radioactivity concentrations in blood and plasma (semilogarithmic view).



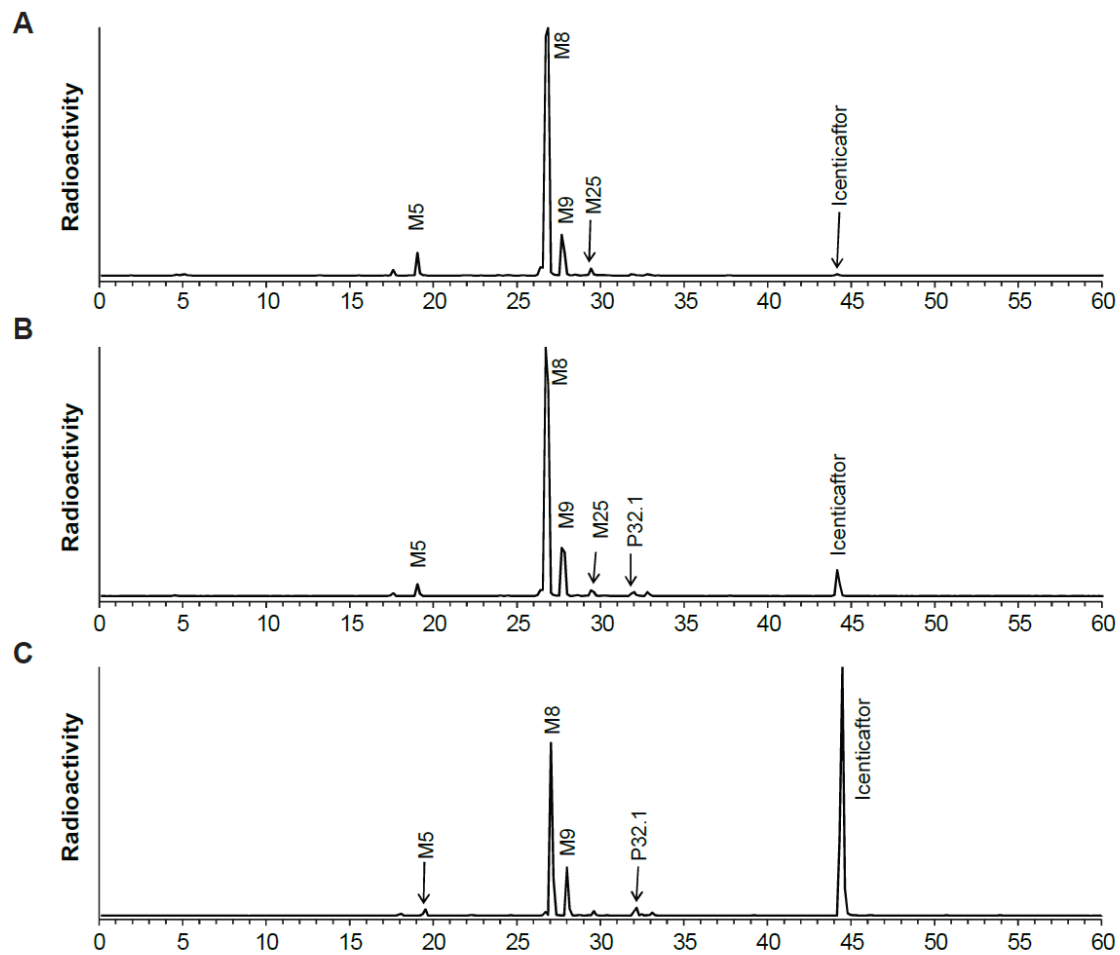
Supplemental Fig. 5. Scheme of the minor biotransformation pathways of icenticaftor in humans (Pathway II).

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

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

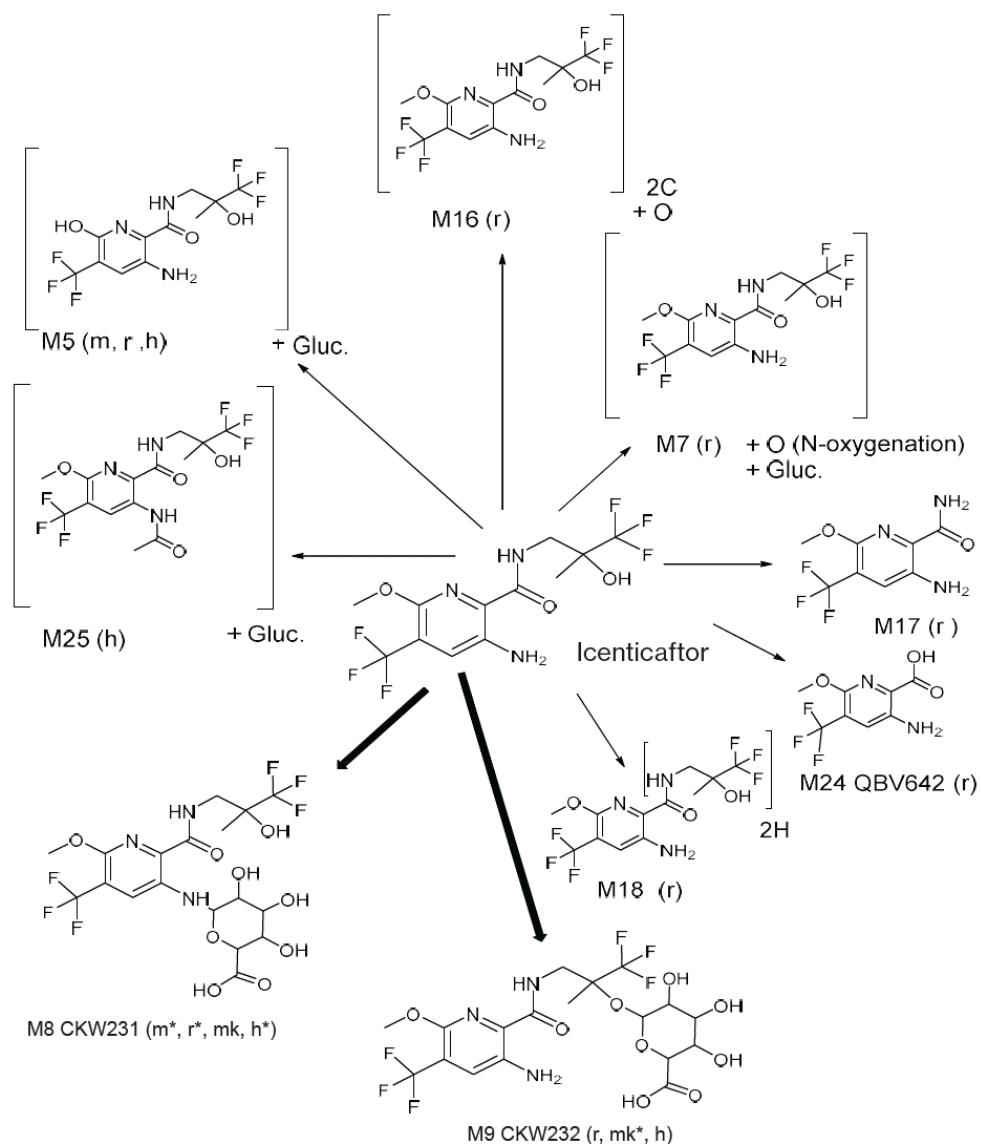


Supplemental Fig. 6. Metabolite profiles of [^{14}C]icenticaftor in human hepatocytes at concentrations of (A) 1 μM , (B) 10 μM , and (C) 30 μM at 24 hours.



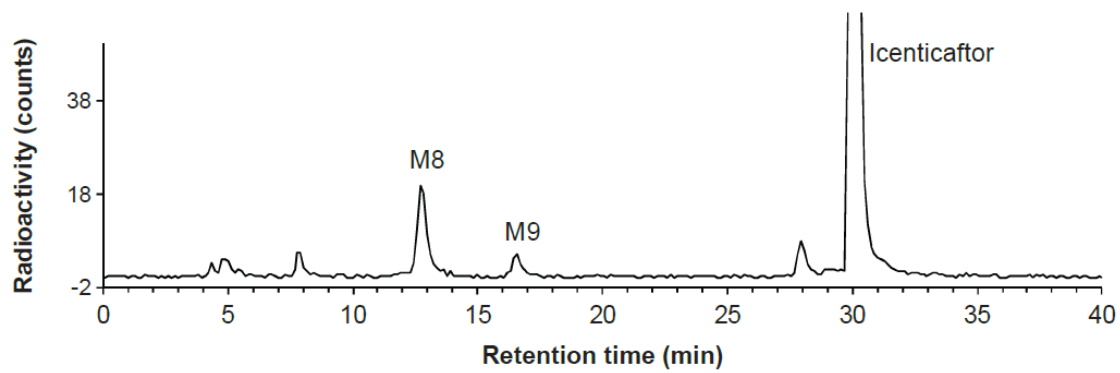
Supplemental Fig. 7. In vitro metabolic pathways across species.

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



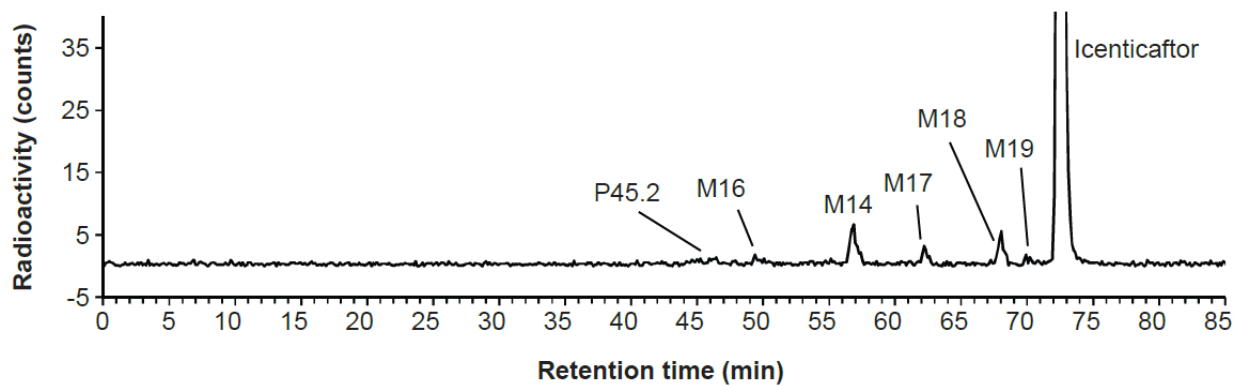
Supplemental Fig. 8. Radiochromatogram of [^{14}C]icenticaftor after incubation for 20 minutes in HLMs in the presence of UDPGA.

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 μ M and 20 μ M icenticaftor in microsomal incubations was performed in triplicates by ultracentrifugation. Values are presented as mean \pm standard deviation of $f_{u,mic}$ for 1 μ M and 20 μ M 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 microsomes.

