THE METABOLISM OF LUFOTRELVIR, A PRODRUG INVESTIGATED FOR THE
TREATMENT OF SARS-COV2, IN HUMANS FOLLOWING INTRAVENOUS
ADMINISTRATION

Narayan Cheruvu, Esther van Duijn, Pieter A. Spigt, Ioana M. Barbu, Sima S. Toussi, Klaas
Schildknegt, Rhys M. Jones, and R. Scott Obach

Pfizer Worldwide Research, Development and Medical, Groton, CT (RSO, KS), LaJolla, CA
(RMJ), Collegeville, PA (NC), Pearl River, NY (SST), and The Netherlands Organization for
Applied Scientific Research (TNO), Zeist, Netherlands (EvD, PAS, and IMB)
Running Title: Metabolism of Lufotrelvir in Humans

Address for Correspondence

R. Scott Obach

Pfizer Inc.

Groton, CT, 06340

r.scott.obach@pfizer.com

860-441-6122

Number of:

Text Pages: 23

Tables: 4

Figures: 6

References: 16

Words in Abstract: 156

Words in Introduction: 756

Words in Discussion: 973

Abbreviations: AMS, accelerator mass spectrometry
ABSTRACT

The metabolism of lufotrelvir, a novel phosphate prodrug of PF-00835231 for the treatment of COVID-19, was evaluated in healthy human volunteers and clinical trial participants with COVID-19 following intravenous infusion. The prodrug was completely converted to PF-00835231 which was subsequently cleared by hydrolysis, hydroxylation, ketoreduction, epimerization, renal clearance, and secretion into the feces. The main circulating metabolite was a hydrolysis product (M7) which was present at concentrations greater than PF-00835231, and this was consistent between healthy volunteers and participants with COVID-19. Upon administration of [14C]lufotrelvir, only 63% of the dose was obtained in excreta over 10 days and total drug-related material demonstrated a prolonged terminal phase half-life in plasma. A considerable portion of the labelled material was unextractable from fecal homogenate and plasma. The position of the carbon-14 atom in the labelled material was at a leucine carbonyl, and pronase digestion of the pellet derived from extraction of the fecal homogenate showed that [14C]leucine was released.
SIGNIFICANCE STATEMENT

Lufotrelvir is an experimental phosphate prodrug intravenous therapy investigated for the potential treatment of COVID-19 in a hospital setting. The overall metabolism of lufotrelvir was determined in human healthy volunteers and clinical trial participants with COVID-19. Conversion of the phosphate prodrug to the active drug PF-00835231 was complete and the subsequent metabolic clearance of the active drug was largely via amide bond hydrolysis. Substantial drug-related material was not recovered due to loss of the carbon-14 label to endogenous metabolism.
INTRODUCTION

In late 2019, one of the greatest threats to the health of humankind, the COVID-19 pandemic, emerged from a new beta coronavirus variant, with evidence strongly supporting a hypothesis of interspecies cross-over to humans in an open-air live animal market in Wuhan, China (Worobey, et al., 2022). The disease manifested as a severe pneumonia and within a year rapidly spread to the rest of the world with hundreds of millions infected, many severely, resulting in a death toll in the millions (World Health Organization, https://covid19.who.int/, accessed December 31, 2022) along with many survivors suffering from a chronic syndrome even after active infection is no longer evident (Yong, 2021). Revolutionary vaccines based on mRNA technology were developed and introduced in early 2021 (Polack, et al., 2020, Baden, et al., 2021) and these have proven effective at reducing the incidence and severity of COVID-19. However as infections continue, particularly in un- and under-vaccinated individuals, new mutants of the virus have arisen (Fernandes, et al., 2022) essentially converting the pandemic into an endemic disease that will continue to cause suffering and death and require ongoing development of new vaccines that target variants as they emerge.

In addition to vaccines, several efforts were made to develop therapies, including antibodies and drugs. Antibodies target the surface proteins of the virus and as new mutants have emerged these treatments have become less effective (Takashita, et al., 2022). The greatest promise for COVID-19 treatment lies in drugs that target specific viral proteins essential for replication and propagation. Early in the pandemic, efforts in our laboratories focused on the development of inhibitors of the main protease of COV-2 to be administered by the intravenous (Boras, et al., 2021) or oral routes (Owen, et al 2021). Success was realized with the design and development
of nirmatrelvir as an oral agent, coadministered with the cytochrome P4503A4 inhibitor ritonavir in Paxlovid™ (Hammond, et al., 2022). This oral treatment was introduced in the United States early in 2022 through Emergency Use Authorization to treat mild-to-moderate COVID-19 in high-risk patients 12+, weighing at least 40 kg, with positive results of SARS-CoV-2 viral testing (see safety info: www.COVID19oralRx.com) and was recently approved by the USFDA.

The efforts at generation of an intravenous agent, with the intent for treatment to occur in hospital settings for patients that cannot receive oral therapy, resulted in the design of lufotrelvir (a.k.a. PF-07304814; Boras, et al., 2021). Lufotrelvir is a phosphate prodrug of PF-00835231 (Figure 1); the latter is a compound that was designed about twenty years ago for potential use in the first coronavirus outbreak (SARS-COV-1). At the time the compound was ready for evaluation in the clinic, the SARS-COV-1 outbreak had been contained and development of PF-00835231 was halted as it was no longer possible to develop it further. Since the main proteases of the coronaviruses causing SARS-COV-1 and SARS-COV-2 are highly conserved, PF-00835231 was deemed a good lead for COVID-19 therapy. However, PF-00835231 did not exhibit adequate oral bioavailability and its aqueous solubility was not amenable for formulation for intravenous dosing. Thus, the approach of a making a soluble phosphate prodrug was pursued, resulting in lufotrelvir as an investigational drug candidate. Lufotrelvir was administered to clinical trial participants and healthy volunteers to explore its pharmacokinetics (Zhu, et al., 2022). It was shown to rapidly and extensively undergo phosphate hydrolysis to yield systemic concentrations of the active PF-00835231 that are high enough to afford good inhibition of the target protease during continuous infusion.
The metabolism of lufotrelvir to PF-00835231 in vitro was shown to be rapid and complete in human liver S9 fraction and further metabolism of the active drug PF-00835231 was demonstrated to be catalyzed by cytochrome P4503A4 in human liver microsomes (Boras, et al., 2021). The objective of the present studies is to evaluate the metabolism and excretion of lufotrelvir and PF-00835231 in humans following intravenous administration, and to compare metabolite profiles in healthy volunteers and individuals with COVID-19. Accelerator mass spectrometry was used as a technology for generating quantitative excretion data because this allowed the administration of a microtracer dose of carbon-14 labelled lufotrelvir. Microtracer approaches obviate the need for a rat tissue distribution study and tissue dosimetry estimations that are prerequisites for dosing amounts of carbon-14 needed for radiometric quantitation. Lufotrelvir was challenged with a need for extremely rapid drug development timelines due to the urgency of the COVID-19 pandemic, so employment of a microtracer/AMS approach to human ADME was essential. Findings are described herein, including experiments done in response to the observation of low recovery of the dose.
METHODS

Materials. Lufotrelvir (PF-07304814), PF-00835231, their epimers, and metabolite M7, were prepared at Pfizer (Hoffman, et al., 2020; Bezawada, et al., 2021). Metabolite M9 was purchased from Aurum Pharmatech (Plano, TX). $[{^{14}}C]\text{Lufotrelvir}$ was prepared under contract by Perkin-Elmer Inc. (Boston, MA) using the synthetic procedures described in the Supplemental Information (Allais, et al., 2023). Pronase was purchased from Sigma (St. Louis, MO).

Dosing of Study Volunteers With $[{^{14}}C]\text{- Lufotrelvir}$ and Sample Collection. Five healthy male and female (of non-childbearing potential only) participants were included in this study. All participants received a single 24 hr constant-rate, continuous IV infusion of 500 mg lufotrelvir containing approximately 420 nCi $[{^{14}}C]\text{- lufotrelvir}$. Serial blood samples were collected at specified times up to 216 hr post the start of the infusion. Total urine collections were made just before dosing (“blank”) and in 24 hr intervals until the end of the study. Feces were collected from Day 1 (“blank”), and at the times of passing while the participant was confined in the clinic. Daily sample collections continued through the morning of discharge. Participants were confined from at least 24 hr prior to dosing on Day 1 until at least the morning of Day 10 (216 hr) post-infusion start. Studies were carried out with informed consent and aligned with the 2018 Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects.

Analysis of Plasma and Urine from Volunteers That Received Non-Labelled Lufotrelvir. Plasma and urine samples were obtained from volunteers with and without COVID-19 that received lufotrelvir infusions in a previously reported study (Zhu, et al., 2022). To individual plasma
samples (0.5 mL) was added acetonitrile (2.5 mL) followed by vigorous mixing on a vortex mixer. The mixtures were spun in a centrifuge for 5 min at 1800 × g and the supernatant transferred to a 15-mL conical glass tube. The liquid was removed by vacuum centrifugation in a Genevac model EZ-2 evaporator at 40°C. The residue was reconstituted in 0.1% formic acid containing 20% acetonitrile (0.1 mL) for analysis by UHPLC-UV-HRMS. To 0.2 mL of pooled urine samples was added 0.05 mL acetonitrile and 0.002 mL formic acid. The mixtures were spun in an Eppendorf microfuge at 13100 x g and the supernatant was transferred for analysis by UHPLC-UV-HRMS.

The analytical instrumentation consisted of a Thermo Vanquish UHPLC with column heater, diode array detector, and refrigerated autosampler maintained at 10°C, coupled in-line with a Thermo Orbitrap Elite ion trap mass spectrometer (Thermo, Waltham, MA). The analytical column was a Kinetex XB-C18 (2.1 × 100 mm, dp = 2.3 µm; Phenomenex, Torrance, CA) maintained at 45°C. Injection volumes were 0.015 mL. The mobile phase was pumped at 0.4 mL/min and was comprised of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). The HPLC pump gradient program began at 95%A/5%B which was held for 0.5 min followed by a linear gradient to 40%A/60%B at 11 min, a second linear gradient to 5%A/95%B at 13 min, held at this composition for 1 min, and then reequilibrated at initial conditions for 2 min. The eluent passed through the diode array UV/VIS detector set at a range of 200-400 nm and was introduced into the HESI source of the mass spectrometer operated in the positive ion mode with source heater temperature of 345°C, potential of 4kV, capillary temperature of 275°C, and sheath, auxiliary, and sweep gas settings at 50, 10, and 2, respectively. The range of first stage scanning was from m/z 100-800 at a resolution setting of 30000. Collision induced dissociation was done at energy settings ranging from 35-45 at a resolution setting of 15000.
Analysis of Plasma and Excreta from Volunteers That Received \[^{14}C\]Lufotrelvir. For each sample, 5 μL of plasma, 15 μL urine, or 30 mg of homogenized feces (diluted or undiluted) was transferred to tin foil cups and analyzed for total \(^{14}\text{C}\) by AMS (van Duijn, et al, 2014) on a 1MV multi-element instrument (High Voltage Engineering Europa B.V. Model 4110; Amersfoort, Netherlands). The samples were dried under a stream of nitrogen and subsequently placed in the elemental analyzer, which acted as an autosampler and combustion device for the AMS. Based on this analysis, excreta samples for each of the five subjects were pooled across sampling times to capture >90% of the carbon-14 in that matrix. Then, equal volumes of each of the three matrices from the five subjects were pooled for quantitative metabolite profiling. Pooling of plasma samples was done in two time regions: 0-24 hr (i.e. during infusion) and 24-216 hr (post-infusion) using volumes in proportion to the time interval represented (Hamilton, et al., 1981). To 0.5 mL pooled plasma was added 2.5 mL acetonitrile. After vortex mixing, the sample was centrifuged at 1800 x g for 5 min set at 10 °C. The initial supernatant was saved and the pellet was washed with another 2.5 mL acetonitrile, centrifuged as above and combined with the initial extract. To the combined supernatant was added glycerol (~0.025 mL) and the sample was lyophilized. The residue was reconstituted in 0.1 mL 20/80/0.1 (v/v/v) acetonitrile/water/formic acid for analysis by UHPLC-HRMS-AMS. To the pooled urine sample (0.2 mL) was added 0.05 mL acetonitrile and 0.002 mL formic acid was added. After mixing, the sample was centrifuged for 5 min at 13100 x g at 10°C and the supernatant was analyzed by UHPLC-HRMS-AMS. To pooled fecal homogenate (1:3 w/w feces/MilliQ; 0.25 mL) was added acetonitrile (1 mL). After vortex mixing, the mixture was centrifuged at 1800 x g for 5 min set at 10 °C and the supernatant was retained. The pellet was re-dispersed with water (0.25 mL) and the procedure was repeated twice more. The three supernatants obtained were combined, glycerol (~0.025 mL) was added
and the sample was lyophilized. The residue was reconstituted in 0.25 mL 20/80/0.1 (v/v/v) for UHPLC-HRMS-AMS analysis.

Analysis was done using an Acquity UPLC system (Waters, Milford, MA) coupled to a Q-Exactive mass spectrometer (Thermo; Waltham, MA). The UHPLC system consisted of a quarternary pump, autosampler, column manager, isocratic pump, and fraction collector. A variable ratio splitter was used to split the column effluent to the fraction collector and mass spectrometer. AMS analysis was done off-line on the fractions of the eluent that were collected every five seconds. The analytical column was a Kinetex XB-C18 (2.1 × 100 mm, dp = 2.3 µm; Phenomenex, Torrance, CA) maintained at 45°C. Injection volumes were 0.02 mL for plasma 0-24 hr, urine and fecal homogenate and 0.05 mL for plasma 24-216 hr. The mobile phase was pumped at 0.4 mL/min and was comprised of 10 mM ammonium acetate in water (solvent A) and acetonitrile (solvent B). The UHPLC pump gradient program began at 100%A which was held for 0.5 min followed by a linear gradient to 78%A/22%B at 3 min, held at this composition for 5 min more, a second linear gradient to 74%A/26%B at 12 min, a third gradient to 5%A/95%B at 13 min, held at this composition for 2 min, and then reequilibrated at initial conditions for 2 min. An additional post-column flow of 0.4 mL/min was introduced to facilitate accurate flow splitting between the Q-Exactive mass spectrometer and the fraction collector (split: plasma 0-24 hr: 72.4% to fraction collector; plasma 24-216 hr and fecal homogenate 79.9% to fraction collector; urine: 79.9% to fraction collector).

Digestion of Solid Pellets and Analysis of Digests by HPLC-AMS. To the pellet obtained after protein precipitation was added 1 mL of 2 mg/mL pronase solution (50 mM ammonium hydrogen carbonate buffer). After vortex mixing the sample was incubated overnight (>16 hr) at 37°C. Afterwards 1 mL acetonitrile was added and the sample was centrifuged for 10 min at
1500 x g at 10°C. The supernatant was evaporated to an approximate volume of 0.2 mL (proteolyzed sample). The Waters AccQ-tag Ultra derivatization kit was employed to derivatize the amino acids. The AccQ-Tag reagent powder was dissolved in 1 mL acetonitrile (15 min, 55°C). To 0.05 mL of the proteolyzed sample 0.35 mL of borate buffer and 0.1 mL of the AccQ-Tag reagent solution were added. After vortex mixing the sample was heated for 10 min at 55°C. The proteolyzed and derivatized plasma pool and fecal homogenate pool samples were analyzed by UHPLC-HRMS with fraction collection for off-line AMS analysis.

The analytical column was a Acquity UPLC-C18 HSS T3 (2.1 × 150 mm, Waters, Milford, CA) maintained at 45°C. Injection volumes were 0.05 mL for both plasma and fecal homogenate samples. The mobile phase was pumped at 0.6 mL/min and was comprised of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The UHPLC pump gradient program began at 96%A which was held for 0.5 min followed by a linear gradient to 90%A/10%B at 2.5 min, a second linear gradient to 72%A/28%B at 12 min, a third gradient to 5%A/95%B at 13 min, held at this composition for 1 min, and then reequilibrated at initial conditions for 1.5 min. The flow splitting between the Q-Exactive mass spectrometer and the fraction collector was 81.8% to the fraction collector). Fractions were collected every 15 seconds for 0-12 min, every 10 seconds for 12-14 min and every 15 seconds for 14-20 min.
RESULTS

*Excretion and Plasma Total Drug-Related Material of Labelled Lufotrelvir.* In five volunteers intravenously infused 500 mg lufotrelvir containing 419 nCi as a microtracer, the overall recovery of carbon-14 labelled material was 63.3% (+/-6.9%; Table 1). The low recovery was consistent across the individuals. The time course of excretion was such that excretion rate was considerably reduced by two days after completion of the infusion (Figure 2). The total drug-related material in plasma is shown in Figure 2 and is marked by a rise during the infusion period, a rapid decline over the next 48 hr and a prolonged terminal phase with a half-life that exceeded 200 hr. A hypothesis for the low recovery and extended half-life of drug-related material is offered in the Discussion (see below).

*Metabolite Profile for Lufotrelvir in Plasma and Urine of Volunteers with COVID-19 and Healthy Volunteers.* Plasma from volunteers with COVID-19 and healthy volunteers receiving non-labelled lufotrelvir by intravenous infusion was evaluated for metabolites using UHPLC-UV-HRMS, and urine from healthy volunteers was also assessed. Example UV chromatograms are shown in Figure 3, and peaks represent drug-related entities that retained the indole moiety. In plasma, the main drug-related materials were the active drug PF-00835231 and its epimer, metabolite M7 which arises via hydrolysis of the P1-P2 amide bond, metabolite M8 which is the O-demethylated analogue of M7, and metabolite M9 (4-methoxyindole-2-carboxylic acid). (Structural assignment information is below.) The metabolite profiles from healthy volunteers and volunteers with COVID-19 did not differ, with the hydrolysis product M7 as the dominant drug-related entity. Lufotrelvir itself was only detected by mass spectrometry indicating much lower levels in circulation than the active drug. In urine, these same metabolites were observed (Figure 3), along with three others: the acyl glucuronide conjugates of M7 and M9 (referred to as
M11 and M10, respectively) and a metabolite designated by its protonated molecular ion of m/z 287.

The proton on the P1 carbon adjacent to the ketone is weakly acidic and thus has the potential to dissociate/reassociate and cause epimerization of the S-configuration of PF-00835231 to the R-isomer. In the UV chromatograms of plasma extracts, the epimer peak was observed to be approximately two-thirds the intensity of PF-00835231, however in UV chromatograms of urine the epimer was considerably smaller than PF-00835231. It was subsequently discovered that the epimerization was dependent on the temperature used during vacuum centrifugation of extracts and the presence of extraneous materials. Urine was not subjected to vacuum centrifugation and thus little epimerization occurred. This information was leveraged in subsequent analyses of samples obtained in the carbon-14 portion of the study wherein lyophilization was used to concentrate extracts instead of vacuum centrifugation to limit epimerization. Thus, while some epimerization of PF-00835231 in vivo is possible, the presence of large amounts of the epimer is an artifact of sample preparation.

**Metabolite Profile for Lufotrelvir in Plasma and Excreta of Healthy Volunteers Quantitated by Accelerator Mass Spectrometry.** Plasma for five volunteers that received a 24 hr infusion of 500 mg lufotrelvir with 419 nCi microtracer was pooled, extracted, and the extract evaluated for metabolite profiles by UHPLC separation, fractionation, and analyzing the fractions by AMS (Figure 4). Simultaneously acquired HRMS data was used for compound identification. Separate plasma pools were prepared for the infusion (0-24 hr) and post-infusion (24-216 hr) periods. Extraction efficiency was low with 60 and 80% of the carbon-14 remaining in the pellet in the 0-24 and 24-216 hr pools. During the infusion, the main drug-related entities in the extract were PF-00835231 and a peak that contained both metabolite M7 and unchanged lufotrelvir that had
coeluted (Table 2). After the infusion, the major metabolite was M7 and there was no unchanged lufotrelvir detected by MS. Because these samples were lyophilized instead of being subjected to vacuum centrifugation, there was little epimer of PF-00835231 observed. As with the earlier non-\textsuperscript{14}C analysis, metabolite M8 was observed, however M9 was not since the labelled carbon atom is not present in M9. Metabolite M6a, arising via reduction of the ketone, was also detected in these samples.

In urine, which had 25.2\% of the dose, the main drug-related entities were PF-00835231 and metabolite M7 (Table 3; Figure 4), along with M8 (O-desmethyl of M7), M6, \textit{m/z} 287, M12 (sulfate ester of M8), and M11 (glucuronide conjugate of M7) which was present as four detectable peaks indicative of acyl glucuronide rearrangement. There were other minor metabolites as well.

Feces contained 38.1\% of the dose and upon sample processing only 63.3\% of the carbon-14 was obtained in the combined extracts, with the remainder in the pelleted solids (Table 3). In the extract, the main drug-related entities eluted in or near the void volume in addition to metabolites M1, M6, and M7 (Figure 4). The fractions at the void and at 1.2 min showed the presence of a predominant ion at \textit{m/z} 132.1019 which is consistent with a molecular formula for leucine (see below).

\textit{Identification of Metabolites by Mass Spectrometry}. Structural assignments for metabolites were made from high resolution mass spectrometry and are summarized on Table 4, with mass spectral data included in the Supplemental Information. Structures are shown in the proposed overall metabolism scheme in Figure 5. Metabolites of unambiguous regiochemical structure
assignments are designated with M numbers while the rest are designated by their protonated molecular ions.

*Lufotrelvir and PF-00835231.* Lufotrelvir had a protonated molecular ion at \( m/z \) 553.2046 consistent with its formula of \( \text{C}_{24}\text{H}_{34}\text{N}_{4}\text{O}_{9}\text{P} \). The fragment ion at \( m/z \) 267 represents cleavage at the P1-P2 amide yielding the 3-amino-2-oxo-4-pyrrolidonyl-butyl phosphate portion, while the ion at \( m/z \) 169 is the same portion without the phosphate. The ion at \( m/z \) 174 contains the methoxyindole portion while \( m/z \) 152 and 124 represent the 2-amino-4-hydroxy-3-oxobutyl pyrrolidinone portion. Lufotrelvir was observed in plasma during the infusion but not in excretory matrices. PF-00835231, the pharmacologically active drug, was observed in all matrices as a major component. It had a protonated molecular ion of \( m/z \) 473.2378 reflective of a formula of \( \text{C}_{24}\text{H}_{33}\text{N}_{4}\text{O}_{6} \). The product ion scan of PF-00835231 yielded diagnostic fragment ions at \( m/z \) 174, 259, and 287 which represent the portion containing the methoxyindole with increasing portions of the leucine P2 attached. Fragment ions at \( m/z \) 187, 152, and 124 represent the 2-amino-4-hydroxy-3-oxobutyl pyrrolidinone portion. Thus, assignments of positions of modification on this core structure can be made using corresponding ions from similar fragmentation reactions. As expected, the epimer of PF-00835231 had the same fragmentation pattern. All subsequent drug-related entities were derived from PF-00835231; there were no metabolites of lufotrelvir wherein the phosphate was retained in the structure.

*Metabolites Arising Via Oxidation and Reduction Reactions.* Metabolism of PF-00835231 occurred by hydroxylation of the 5-position of the pyrrolidinone (M1). M1 was observed in the fecal extract and had a protonated molecular ion of \( m/z \) 489.2335 indicating the addition of one oxygen atom. The product ion scan of M1 yielded diagnostic fragment ions with \( m/z \) 174, 259, and 287 supporting no change to the indole/leucine portion and \( m/z \) 203 and 140 indicating the
position of hydroxylation in the 2-amino-4-hydroxy-3-oxobutyl pyrrolidinone portion. A comparison of the retention time and fragmentation pattern for this peak in the fecal extract matched the metabolite previously observed in HLM and CYP3A4 (Boras, et al., 2021) and supports the assignment specifically as M1.

Metabolite M6a was observed in plasma, urine, and fecal homogenate and had a protonated molecular ion of m/z 475.2539 consistent with an empirical formula of C_{24}H_{35}N_{4}O_{6} representing the addition of two hydrogen atoms. The product ion scan of M6a yielded diagnostic fragment ions of m/z 174 (no change to the indole) and 189 which shows addition of 2H to the 2-amino-4-hydroxy-3-oxobutyl pyrrolidinone portion. The ion at m/z 136 is proposed to arise via dehydration of a minor ion at m/z 154, which itself is analogous to the ion at m/z 152 observed for PF-00835231. This all supports the putative assignment of M6a as a metabolite arising from reduction of the ketone in PF-00835231. Another metabolite (M6b) with the same molecular ion and fragment ions at m/z 189 and 174 was also observed in much lower apparent abundance in excreta. It is proposed as a diastereomer of M6a, as ketoreduction has the potential to generate alcohols of both configurations. The exact stereochemistry of the hydroxyl group in M6a and M6b arising from ketoreduction is not known.

M1 and/or M6a and M6b appeared to undergo subsequent metabolism to generate three minor metabolites with protonated molecular ions at m/z 491 that were observed in fecal homogenate extracts. One (m/z 491a) eluted earlier in the chromatogram than the other two. The earlier eluting metabolite (which coeluted with another metabolite at m/z 487) had a pronated molecular ion of m/z 491.2496 consistent with an empirical formula of C_{24}H_{35}N_{4}O_{7} and the addition of an oxygen and two hydrogen atoms to PF-00835231. Fragment ions at m/z 189, 172, and 136 support that the addition of two hydrogens is from ketoreduction, analogous to M6. The
fragment ion at m/z 174 indicates that the indole is unmodified. By process of elimination, the site of hydroxylation is proposed to be on the leucine portion.

The other two metabolites (m/z 491b and 491c) were observed in feces with protonated molecular ions at m/z 491.2502 which is also consistent with the addition of two hydrogen atoms and one oxygen atom. The fragmentation pattern for these two metabolites was nearly identical. As with m/z 491a, the fragment ion at m/z 174 shows no change to the indole portion. The minor fragment ion at m/z 205 suggests all modification to have occurred on the 2-amino-4-hydroxy-3-oxobutyl pyrrolidinone portion with the major ion at m/z 187 resulting from dehydration of m/z 205. These two metabolites could have arisen via hydrolysis of the pyrrolidone ring on PF-00835231 itself or they could be hydroxy metabolites of M6a and M6b; the MS data does not provide enough evidence to favor one proposal over the other.

Finally, two minor metabolites were observed in fecal homogenates with protonated molecular ions of m/z 487.2188 and 487.2187 consistent with an empirical formula of C_{24}H_{31}N_{4}O_{7}. This represents the addition of an oxygen to and loss of two hydrogen atoms from PF-00835231. The protonated molecular ions were low relative to background, however the fragmentation patterns consistently yielded m/z 174 which indicates no change to the methoxyindole portion. The modifications are proposed to have occurred on the 2-amino-4-hydroxy-3-oxobutyl pyrrolidinone portion due to the presence of m/z 201, which is 14 mass units higher than the corresponding diagnostic fragment ion at m/z 187 for PF-00835231. The exact modifications (i.e. whether the dehydrogenation results in a succinimide or hydroxy/alkene metabolite) cannot be determined from the MS data alone.
Metabolites Arising Via Hydrolysis Reactions. The main metabolite of PF-00835231 in plasma and urine is M7 with a protonated molecular ion at m/z 305.1493 yielding an empirical formula of C_{16}H_{21}N_{2}O_{4}. With this protonated molecular ion, the structure assignment is unambiguous. M8 was observed in plasma and urine and had a protonated molecular ion of m/z 291.1336 consistent with an empirical formula of C_{15}H_{19}N_{2}O_{4}. With this mass, it can only be the O-desmethyl metabolite of M7 and the main fragment ion of m/z 160 represents the indole portion that has been demethylated. Mass spectral data that would be consistent with O-desmethyl PF-00835231 was not observed allowing inference that M8 arises from M7 and not via hydrolysis of an O-desmethyl metabolite of PF-00835231.

Metabolite M9 was observed as a peak in the UV chromatograms from all volunteers (Figure 3), but in the carbon-14 part of the study this metabolite would not be observable by AMS since the labelled atom is lost. It possesses a protonated molecular ion of m/z 192.0655 which is consistent with an empirical formula of C_{10}H_{10}NO_{3} (-0.1 ppm) and limits the possibilities to a structural assignment to 4-methoxy-1H-indole-2-carboxylic acid. The presence of the carboxylic acid is supported by the fragment ion loss of 44 mass units (-CO_{2}).

Hydrolysis products M7, M8, and M9 all underwent further metabolism by conjugation reactions. M10, with a protonated molecular ion 368.0975, is proposed as the glucuronide conjugate of M9. It was observed in the UV chromatogram of urine but not in the AMS chromatogram, consistent with the fact that it does not possess the labelled carbon atom in [^{14}C]lufotrelvir. Urinary metabolite M11 showed four chromatographic peaks with the same protonated molecular ion of m/z 481.1812 which yields a molecular formula of C_{22}H_{29}N_{2}O_{10} and the fragment ion of m/z 305 is consistent with assignment as a glucuronide conjugate of M7. The presence of four peaks suggests rearrangement of the acyl glucuronide. Metabolite M12 was
present in plasma and urine and had a protonated molecular ion of \(m/z\) 371.0908 which is consistent with assignment as a sulfate conjugate of M8. M8 also yields two glucuronide conjugates (\(m/z\) 467a and b), however fragmentation data did not permit assignment of the carboxylic acid or phenol as the site(s) of conjugation.

The minor urinary metabolite \(m/z\) 287 yielded a high resolution protonated molecular ion of 287.1387 which is consistent with M7 and a loss of water. The structure is proposed as arising via an intramolecular cyclization of the indole nitrogen and carboxyl moiety of M7 to yield a dihydropyrazino[1,2-a]indole-1,4-dione ring system. It may also have arisen via intramolecular cyclization of M11, with elimination of glucuronic acid. Some spectral fragmentation evidence supports, but does not prove, this assignment (see proposed structures for fragment ions in the Supplemental Information).

Finally, in the AMS chromatogram from the fecal homogenate extract there were two peaks eluting in the column void volume and just after the void volume (Figure 4). A predominant ion at \(m/z\) 132.1019 was observed and this suggests a molecular formula of \(C_6H_{14}NO_2\) which is the same formula for leucine (as well as isoleucine). In the reconstructed ion chromatogram for \(m/z\) 132.1019 (to a tolerance of five ppm), the same pattern of a large peak in the void volume followed by a smaller peak at 1.2 min emerged. A standard of leucine eluted at 1.2 min. With the observation of the hydrolysis product M9, the release of labelled leucine (the P2 portion) is plausible if M9 arises from the major metabolite M7. The presence of the \(m/z\) 132.1019 ion in the void volume could be due to the presence of labelled leucine within a larger peptide and \(m/z\) 132.1019 arising via in-source fragmentation. This cannot be distinguished because leucine is present in control fecal homogenate without dosing of lufotrelvir, since it is an endogenous amino acid.
Investigation of Low Mass Balance and Unextractable Drug-Related Material. As described above, analysis of excreta and plasma showed low mass balance, an extended half-life of carbon-14 in plasma, and retention of carbon-14 in solids remaining after miscible organic extraction of plasma and fecal homogenates. The fecal and plasma pellets were subject to digestion with pronase and the digest was fractionated by UHPLC with the fractions analyzed by AMS. The plasma pellet digestion did not yield enough signal for AMS however the fecal pellet did (Figure 6). While a considerable portion of carbon-14 eluted in the void volume which may indicate incomplete digestion, a peak was also observed at the retention time of derivatized leucine (13 min). This suggests that [14C]leucine was generated as a metabolite of [14C]lufotrelvir through hydrolysis (as suggested above) and that this entered the endogenous amino acid pool where it was subsequently incorporated into macromolecules. A second peak eluting at approximately 16 min corresponded to PF-00835231. It is important to note that PF-00835231 exhibits its action on the viral protease by forming a reversible covalent bond between the ketone carbon and an active site cysteine (Boras, et al., 2021). Thus, this material in the fecal pellet digest could be PF-00835231 that was bound to proteins and released during the sample processing.
DISCUSSION

The overall disposition of \([^{14}C]\)lufotrelvir has now been determined in healthy volunteers and is characterized by complete conversion of the phosphate prodrug to the active drug PF-00835231. When tracked through the labelled carbon on the P2 leucine, PF-00835231 underwent subsequent clearance by (1) oxidative and reductive metabolism (~5%), (2) hydrolytic metabolism (~20%), (3) urinary clearance (~8%), (4) fecal clearance either through biliary elimination or intestinal secretion (<1%), (5) epimerization (~1-2%), and (6) incorporation into endogenous metabolism (~60-70%; estimated by missed mass balance and unextractable material in the fecal pellet). Data were collected to suggest that latter occurred through hydrolysis with the \([^{14}C]\)leucine released becoming incorporated into endogenous metabolism, supporting that hydrolysis is the major clearance pathway for PF-00835231.

Circulating metabolite profiles were similar between volunteers with COVID-19 and healthy volunteers. During infusion, some lufotrelvir itself was observed but much greater levels were seen for the active drug PF-00835231. Also, lufotrelvir was not observed in excreta. This indicates that dephosphorylation to the active drug occurs extensively and rapidly, consistent with previous observations (Zhu, et al., 2022). Individuals with COVID-19 suffer from inflammation, which can affect drug metabolism (Stanke-Labesque, et al., 2020), but this did not appear to have any meaningful impact on metabolism of PF-00835231. The main circulating metabolite in volunteers with COVID-19 and healthy volunteers was M7, arising from hydrolysis of the P1-P2 amide bond.

The proclivity for epimerization of the carbon adjacent to the ketone offered a technical challenge. Initial evaluations of metabolic profiles in plasma showed considerable amounts of
the epimer of PF-00835231 and it was not known if epimerization had occurred in vivo. In urine samples, the epimer was minor suggesting either that PF-00835231 and its epimer undergo very different levels of renal secretion (unlikely) or that the epimer seen in plasma was an artifact caused by sample processing. For analysis of metabolite profiles, sample processing for plasma included a concentrative step to permit gathering quality HPLC-UV data, but urine could be analyzed by HPLC-UV without a concentration step. Concentration of plasma extracts (mixtures of acetonitrile and aqueous) was done by vacuum centrifugation at ~40°C and it was shown that this step of the process caused epimerization. When plasma extracts were instead processed using lyophilization, the epimerization was considerably abated. Thus, epimerization is a minor pathway for PF-00835231 clearance.

The COVID-19 pandemic triggered substantial investment and effort in the development of vaccines and therapeutic agents with a great sense of urgency. To expedite the human ADME study, AMS technology was leveraged which, due to sensitivity that is several orders of magnitude greater than liquid scintillation counting, permits administration of very low quantities of labelled material (i.e. a microtracer dose). In this study, a microtracer dose of 419 nCi was administered (in a clinically relevant dose of 500 mg), and thus the study could be expedited without the prerequisite rodent tissue distribution and radioactivity dosimetry information. However, an AMS study still requires generation of carbon-14 labelled drug for human dosing which takes considerable lead time to prepare. For [14C]lufotrelvir, synthesis was expedited by using commercially available [14C]leucine methyl ester resulting in lufotrelvir labelled at the carbonyl carbon of the P2 segment, the only portion of lufotrelvir that is a natural amino acid. Prior to administration to humans, hydrolysis of PF-00835231 to M7 had been demonstrated in vitro in human digestive juice, but hydrolysis reactions were not observed to any
substantial degree in any other human derived in vitro matrices such as plasma or liver S9 fraction (Pfizer, data on file), and M9, which would result from release of leucine from M7, had not been observed. Thus, the risk was taken to place the label on the leucine in lufotrelvir, knowing that if the leucine were released via metabolism, there could be challenges in the study due to incorporation of carbon-14 into endogenous metabolism. When lufotrelvir was administered to humans in phase 1 trials, it was discovered that PF-00835231 did undergo hydrolytic metabolism to a considerable degree, as observed through metabolites M7 and M9 and subsequent conjugates (Figure 3). This observation was borne out in (1) the low mass balance of 63.3% obtained following administration of leucine labelled lufotrelvir, (2) the prolonged half-life of drug-related material in plasma, and (3) carbon-14 that was retained in solids following extraction of plasma and fecal homogenates. Partial digestion of these solids from extracts showed the release of [14C]leucine, and thus the low mass balance can be explained by incorporation of label into endogenous macromolecules. Leucine is also metabolized by transaminases that yield keto acids, which can enter into fatty acid and sterol biosynthesis as well as lose CO₂ (Lee, et al., 2015), thus the carbon-14 label of lufotrelvir had opportunity to enter all sorts of endogenous biomolecules. Placement of the label in the P1 or P3 portions of lufotrelvir may have avoided the confounding observations obtained in the present study. Furthermore, PF-00835231 itself was observed in the fecal pellet that remained after extraction, and this could be due to reversible covalent bonds between the ketone and protein nucleophiles (thiol and amino). It is known that the ketone of PF-00835231 is essential for target activity because it reacts with an active site cysteine, and thus it is reasonable to propose that it could react with other proteins.

Overall, the conclusions from these investigations are that lufotrelvir is extensively dephosphorylated to yield the active agent PF-00835231 which is subsequently mostly cleared
by hydrolytic metabolism, with minor contributions from hydroxylation, ketoreduction, epimerization, and urinary clearance pathways. The enzymes involved in hydrolysis are not known and their identification will require further investigation. Application of AMS technology to this human ADME study accommodated the urgent need for the development of a COVID-19 therapy, however placement of the carbon-14 label in the molecule in the P2 leucine moiety complicated the outcome due to hydrolytic metabolism that released [14C]leucine into endogenous metabolism.
AUTHOR CONTRIBUTIONS:

Participated in research design: Cheruvu, Toussi, Jones, Obach

Conducted experiments: Spigt, Barbu, Obach

Contributed new reagents or analytic tools: Schildknegt

Performed data analysis: van Duijn, Cheruvu, Obach

Wrote or contributed to the writing of the manuscript: van Duijn, Cheruvu, Schildknegt, Obach

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


FIGURE LEGENDS

FIGURE 1. Structure of lufotrelvir and the active compound PF-00835231. The asterisk indicates the carbon atom that is labeled. The dashed lines indicate the amide bonds that separate the molecules into “peptide” fragment terminology.

FIGURE 2. Cumulative excretion of \([14C]\)lufotrelvir drug-related material over time (left plot) and total drug-related material in plasma vs time (right plot) in human volunteers following intravenous infusion of 500 mg (419 nCi) over 24 hrs. The infusion period is indicated by the blue bar on the x-axis.

FIGURE 3. Example UHPLC-UV chromatograms of plasma extracts and urine from healthy volunteers and volunteers with COVID-19 following intravenous administration of lufotrelvir.

FIGURE 4. UHPLC-AMS chromatograms of pooled plasma extracts, pooled urine, and pooled fecal extracts from healthy volunteers following intravenous administration of \([14C]\)lufotrelvir.

FIGURE 5. Proposed overall metabolic scheme for lumotrelvir in humans.

FIGURE 6. UHPLC-AMS chromatogram of the pronase digest of the fecal pellet.
FOOTNOTES

This work received no external funding. NC, SST, KS, RMJ and RSO are employees and shareholders of Pfizer Inc.
TABLE 1. Mass Balance Excretion of $[^{14}C]Lufotrelvir$ in Healthy Male Volunteers Following Intravenous Infusion of 500 mg.

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>% of Dose in Urine</th>
<th>% of Dose in Feces</th>
<th>% of Dose Excreted</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>28.7</td>
<td>33.0</td>
<td>61.7</td>
</tr>
<tr>
<td>B</td>
<td>23.1</td>
<td>41.2</td>
<td>64.2</td>
</tr>
<tr>
<td>C</td>
<td>22.0</td>
<td>52.6</td>
<td>74.6</td>
</tr>
<tr>
<td>D</td>
<td>28.9</td>
<td>28.5</td>
<td>57.3</td>
</tr>
<tr>
<td>E</td>
<td>23.2</td>
<td>35.3</td>
<td>58.5</td>
</tr>
<tr>
<td>Mean</td>
<td>25.2</td>
<td>38.1</td>
<td>63.3</td>
</tr>
<tr>
<td>SD</td>
<td>3.3</td>
<td>9.3</td>
<td>6.9</td>
</tr>
</tbody>
</table>
TABLE 2. Summary of Metabolites of $[^{14}\text{C}]$Lufotrelvir in Pooled Plasma of Healthy Volunteers Following Intravenous Administration of 500 mg (410 nCi) as Quantified by AMS.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>T_R (min)</th>
<th>m/z (M+H&lt;sup&gt;+&lt;/sup&gt;)</th>
<th>0-24 hr Pool</th>
<th>24-216 hr Pool</th>
</tr>
</thead>
<tbody>
<tr>
<td>M12</td>
<td>3.1</td>
<td>371.0908</td>
<td>&lt;0.4</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>M8</td>
<td>3.8</td>
<td>291.1336</td>
<td>1.1</td>
<td>0.4</td>
</tr>
<tr>
<td>M7</td>
<td>5.5</td>
<td>305.1493</td>
<td>21.5</td>
<td>12.8</td>
</tr>
<tr>
<td>Lufotrelvir</td>
<td></td>
<td>553.2046</td>
<td></td>
<td>&lt;1</td>
</tr>
<tr>
<td>M6a</td>
<td>10.6</td>
<td>475.2539</td>
<td>&lt;0.4</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>PF-00835231</td>
<td>12.3</td>
<td>473.2378</td>
<td>9.8</td>
<td>2.2</td>
</tr>
<tr>
<td>Epimer</td>
<td>12.7</td>
<td>473.2390</td>
<td>1.3</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Unextracted in Pellet</td>
<td></td>
<td></td>
<td>60.6</td>
<td>80.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Approximately 1% and 1.7% was present in unidentified peaks in the 0-24 and 24-216 hr pools.
TABLE 3. Summary of Metabolites of \[^{14}\text{C}]\text{Lufotrelvir}
 in Pooled Excreta of Healthy Volunteers
Following Intravenous Administration of 500 mg (410 nCi) as Quantified by AMS.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>(T_R) (min)</th>
<th>(m/z) (M+H(^+))</th>
<th>Urine(^a)</th>
<th>Feces(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>leucine in void</td>
<td>0.6</td>
<td>132.1019</td>
<td>nd(^c)</td>
<td>6.2</td>
</tr>
<tr>
<td>leucine</td>
<td>1.2</td>
<td>132.1019</td>
<td>nd(^c)</td>
<td>0.8</td>
</tr>
<tr>
<td>(m/z) 467a</td>
<td>2.5</td>
<td>467.1663</td>
<td>&lt;0.3</td>
<td>nd(^c)</td>
</tr>
<tr>
<td>(m/z) 467b</td>
<td>2.7</td>
<td>467.1661</td>
<td>&lt;0.3</td>
<td>nd(^c)</td>
</tr>
<tr>
<td>M12</td>
<td>3.1</td>
<td>371.0908</td>
<td>1.7</td>
<td>nd(^c)</td>
</tr>
<tr>
<td>M8</td>
<td>3.8</td>
<td>291.1336</td>
<td>2.3</td>
<td>nd(^c)</td>
</tr>
<tr>
<td>(m/z) 491a</td>
<td>4.8</td>
<td>491.2496</td>
<td>nd(^c)</td>
<td>0.4</td>
</tr>
<tr>
<td>(m/z) 487a</td>
<td></td>
<td>487.2188</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(m/z) 487b</td>
<td>5.0</td>
<td>487.2187</td>
<td>nd(^c)</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>M1</td>
<td>5.3</td>
<td>489.2335</td>
<td>nd(^c)</td>
<td>0.8</td>
</tr>
<tr>
<td>M7</td>
<td>5.5</td>
<td>305.1493</td>
<td>5.4</td>
<td>nd(^c)</td>
</tr>
<tr>
<td>M11a-d</td>
<td>6.4-7.7</td>
<td>481.1812</td>
<td>3.6</td>
<td>nd(^c)</td>
</tr>
<tr>
<td>(m/z) 491b</td>
<td>8.6</td>
<td>491.2502</td>
<td>nd(^c)</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>(m/z) 491c</td>
<td>9.4</td>
<td>491.2502</td>
<td>nd(^c)</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>M6a</td>
<td>10.7</td>
<td>475.2539</td>
<td>&lt;0.3</td>
<td>2.6</td>
</tr>
<tr>
<td>M6b</td>
<td>11.4</td>
<td>475.2539</td>
<td>&lt;0.3</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>PF-00835231</td>
<td>12.8</td>
<td>473.2378</td>
<td>7.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Epimer</td>
<td>13.0</td>
<td>473.2390</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>(m/z) 287</td>
<td>13.5</td>
<td>287.1387</td>
<td>0.4</td>
<td>nd(^c)</td>
</tr>
<tr>
<td>Unextracted</td>
<td></td>
<td></td>
<td>n/a(^b)</td>
<td>36.4</td>
</tr>
</tbody>
</table>

\(^a\)Approximately 0.5 and 2.4% was present in unidentified peaks in the urine and fecal extract respectively.

\(^b\)n/a, not applicable

\(^c\)nd, not detected
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>m/z (M+H⁺)</th>
<th>Formula</th>
<th>Δ (ppm)</th>
<th>Fragment Ions</th>
<th>Proposed Transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lufotrelvir</td>
<td>553.2046</td>
<td>C_{24}H_{34}N_{4}O_{9}P</td>
<td>-2.5</td>
<td>456, 267, 174, 169, 152</td>
<td>Parent Compound</td>
</tr>
<tr>
<td>PF-00835231</td>
<td>473.2378</td>
<td>C_{24}H_{33}N_{4}O_{6}</td>
<td>-3.5</td>
<td>287, 187, 169, 152</td>
<td>Hydrolysis of Lufotrelvir</td>
</tr>
<tr>
<td>Epimer</td>
<td>473.2390</td>
<td>C_{24}H_{33}N_{4}O_{6}</td>
<td>-1.0</td>
<td>287, 187, 169, 152</td>
<td>Epimerization of PF-00835231</td>
</tr>
<tr>
<td>Leucine</td>
<td>132.1019</td>
<td>C_{6}H_{14}NO_{2}</td>
<td>-0.04</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>489.2335</td>
<td>C_{24}H_{33}N_{4}O_{6}</td>
<td>0.9</td>
<td>259, 203, 174, 140</td>
<td>Hydroxylation of PF-00835231</td>
</tr>
<tr>
<td>M6a</td>
<td>475.2539</td>
<td>C_{24}H_{34}N_{4}O_{6}</td>
<td>-0.9</td>
<td>458, 289, 189, 174, 136</td>
<td>Ketoreduction of PF-00835231</td>
</tr>
<tr>
<td>M6b</td>
<td>475.2539</td>
<td>C_{24}H_{34}N_{4}O_{6}</td>
<td>-0.11</td>
<td>189, 174</td>
<td>Ketoreduction of PF-00835231</td>
</tr>
<tr>
<td>M7</td>
<td>305.1493</td>
<td>C_{16}H_{21}N_{2}O_{4}</td>
<td>-0.9</td>
<td>174, 161, 119</td>
<td>Hydrolysis of PF-00835231</td>
</tr>
<tr>
<td>M8</td>
<td>291.1336</td>
<td>C_{15}H_{18}N_{2}O_{4}</td>
<td>-1.1</td>
<td>245, 160, 147</td>
<td>O-Demethylation of M7</td>
</tr>
<tr>
<td>M9</td>
<td>192.0655</td>
<td>C_{10}H_{10}NO_{3}</td>
<td>-0.1</td>
<td>174, 148, 133</td>
<td>Hydrolysis of M7</td>
</tr>
<tr>
<td>M10</td>
<td>368.0975</td>
<td>C_{16}H_{18}NO_{9}</td>
<td>-0.3</td>
<td>350, 192, 174, 148</td>
<td>Glucuronidation of M9</td>
</tr>
<tr>
<td>M11</td>
<td>481.1812</td>
<td>C_{22}H_{29}N_{2}O_{10}</td>
<td>-1.0</td>
<td>463, 305, 287</td>
<td>Glucuronidation of M7</td>
</tr>
<tr>
<td>M12</td>
<td>371.0908</td>
<td>C_{13}H_{19}N_{2}O_{7}S</td>
<td>0.3</td>
<td>325, 240, 160, 87</td>
<td>Sulfation of M8</td>
</tr>
<tr>
<td>m/z 287</td>
<td>287.1387</td>
<td>C_{16}H_{19}N_{2}O_{3}</td>
<td>-1.1</td>
<td>259, 231, 216, 202, 174, 148</td>
<td>Cyclization of M7</td>
</tr>
<tr>
<td>m/z 467a</td>
<td>467.1663</td>
<td>C_{21}H_{27}N_{2}O_{10}</td>
<td>0.6</td>
<td>291, 160, 134, 86</td>
<td>Glucuronidation of M8</td>
</tr>
<tr>
<td>m/z 467b</td>
<td>467.1661</td>
<td>C_{21}H_{27}N_{2}O_{10}</td>
<td>0.2</td>
<td>291, 160, 134, 86</td>
<td>Glucuronidation of M8</td>
</tr>
<tr>
<td>m/z 487a</td>
<td>487.2188</td>
<td>C_{24}H_{31}N_{4}O_{7}</td>
<td>0.2</td>
<td>469, 259, 201, 174, 125</td>
<td>Dehydrogenation of M1</td>
</tr>
<tr>
<td>m/z 487b</td>
<td>487.2187</td>
<td>C_{24}H_{31}N_{4}O_{7}</td>
<td>-0.1</td>
<td>469, 259, 201, 174, 125</td>
<td>Dehydrogenation of M1</td>
</tr>
<tr>
<td>m/z 491a</td>
<td>491.2496</td>
<td>C_{24}H_{35}N_{4}O_{7}</td>
<td>-0.9</td>
<td>473, 189, 174, 172, 154, 136</td>
<td>Hydroxylation of M6</td>
</tr>
<tr>
<td>m/z 491b</td>
<td>491.2502</td>
<td>C_{24}H_{35}N_{4}O_{7}</td>
<td>0.4</td>
<td>455, 187, 174, 152</td>
<td>Hydroxylation of M6</td>
</tr>
<tr>
<td>m/z 491c</td>
<td>491.2502</td>
<td>C_{24}H_{35}N_{4}O_{7}</td>
<td>0.4</td>
<td>455, 187, 174, 152</td>
<td>Hydroxylation of M6</td>
</tr>
</tbody>
</table>
Figure 1

Lufotrelvir (prodrug) → PF-00835231 (active drug)
Figure 2

This article has not been copyedited and formatted. The final version may differ from this version.

DMD Fast Forward. Published on July 10, 2023 as DOI: 10.1124/dmd.123.001416

Downloaded from dmd.aspetjournals.org on July 21, 2023
Figure 3

Healthy Volunteer Plasma;
Dose = 500 mg; 24 hr

COVID-19 Patient Plasma;
Dose = 500 mg/day; 120 hr

Healthy Volunteer Urine
Dose = 700 mg; 12-24 hr pool
Figure 6
Supplemental Information for

THE METABOLISM OF LUFOOTRELVIR, A PRODRUG FOR THE TREATMENT OF SARS-COV2, IN HUMANS FOLLOWING INTRAVENOUS ADMINISTRATION

Narayan Cheruvu, Esther van Duijn, Pieter A. Spigt, Ioana M. Barbu, Sima S. Toussi, Klaas Schildknegt, Rhys M. Jones, and R. Scott Obach

Contents

SYNTHESIS OF [14C]LUFOOTRELVIR 2
SUPPLEMENTAL FIGURE 1. Mass Spectra for Lufotrelvir 6
SUPPLEMENTAL FIGURE 2. Mass Spectra for PF-00835231 7
SUPPLEMENTAL FIGURE 3. Mass Spectra for Epimer of PF-00835231 8
SUPPLEMENTAL FIGURE 4. Mass Spectra for Leucine 9
SUPPLEMENTAL FIGURE 5. Mass Spectra for Metabolite M1 10
SUPPLEMENTAL FIGURE 6. Mass Spectra for Metabolite M6a 11
SUPPLEMENTAL FIGURE 7. Mass Spectra for Metabolite M6b 12
SUPPLEMENTAL FIGURE 8. Mass Spectra for Metabolite M7 13
SUPPLEMENTAL FIGURE 9. Mass Spectra for Metabolite M8 14
SUPPLEMENTAL FIGURE 10. Mass Spectra for Metabolite M9 15
SUPPLEMENTAL FIGURE 11. Mass Spectra for Metabolite M10 16
SUPPLEMENTAL FIGURE 12. Mass Spectra for Metabolite M11 17
SUPPLEMENTAL FIGURE 13. Mass Spectra for Metabolite M12 18
SUPPLEMENTAL FIGURE 14. Mass Spectra for Metabolite m/z 287 19
SUPPLEMENTAL FIGURE 15. Mass Spectra for Metabolites m/z 467a and 467b 20
SUPPLEMENTAL FIGURE 16. Mass Spectra for Metabolites m/z 487a and 487b 21
SUPPLEMENTAL FIGURE 17. Mass Spectra for Metabolite m/z 491a 22
SUPPLEMENTAL FIGURE 18. Mass Spectra for Metabolites m/z 491b and 491c 23
The synthesis of $^{14}$C-lufotrelvir IV Specific Activity was performed at PerkinElmer Health Sciences, Inc., (Boston, MA) using experimental procedures obtained from Pfizer Chemical Research & Development Department (Groton, CT) and adapted for use with radiolabeled compounds.
[^14]C)methyl (4-methoxy-1H-indole-2-carbonyl)-L-leucinate (3). A round-bottomed flask was charged with 4-methoxy-1H-indole-2-carboxylic acid (1) (956 mg, 5.00 mmol, 1 eq), [^14]C]leucine methyl ester hydrochloride (2) (ca. 310 mCi, 963 mg, 5.30 mmol, 1.06 eq) and acetonitrile (7 mL). The mixture was stirred at room temperature and N-methylimidazole (1.4 mL, 17.5 mmol) was added dropwise. The resulting solution was treated with T3P (50% in acetonitrile, 4.4 mL) and the reaction was stirred at room temperature for 1.5 hours. Radio-TLC reaction monitoring (silica gel GF; 2:1 hexanes : ethyl acetate) indicated >95% formation of (3). The reaction was filtered through Celite, rinsing with acetonitrile. The filtrate was concentrated to ca. 10 mL under reduced pressure and 1.5 mL of water was added with stirring. The resulting suspension was further diluted with water (8 mL), filtered and collected solids were washed with water and suction dried to provide (3) as an off-white solid in 84% yield (ca. 261 mCi, 1420 mg, 4.46 mmol).

[^14]C](4-methoxy-1H-indole-2-carbonyl)-L-leucine (4). A round-bottomed flask containing a room temperature solution of acetic acid (7 mL), water (1.5 mL) and concentrated sulfuric acid (0.36 mL) was charged with (3) (ca. 261 mCi, 1420 mg, 4.46 mmol). The resulting clear solution was heated to 45 °C under positive nitrogen pressure. After 24 hours radio-TLC reaction monitoring (silica gel GF; 80:20:4 chloroform : methanol : conc. NH₄OH) indicated approximately 2% (3) remaining. Water (10.5 mL) was slowly added to the reaction and after an additional 1 hour the forming suspension was cooled to room temperature. Stirring was continued for 1 hour before the suspension was filtered and the collected solids were washed with water and suction dried to afford (4) as an off-white solid (ca. 207 mCi, 1077 mg, 3.54 mmol) in 79% yield.

[^14]C]methyl (S)-2-((S)-2-(4-methoxy-1H-indole-2-carboxamido)-4-methylpentanamido)-3-((S)-2-oxopyrrolidin-3-yl)propanoate (5). 2-Hydroxypyridine N-oxide (98 mg, 0.88 mmol, 0.25 eq) was added to a round bottomed flask containing a room temperature slurry of (4) (ca. 207 mCi, 1077 mg, 3.54 mmol, 1 eq) and (S)-1-methoxy-1-oxo-3-((S)-2-oxopyrrolidin-3-yl)propan-2-aminium tosylate (1332 mg, 3.72 mmol, 1.05 eq) in methyl ethyl ketone (5.4 mL). The resulting mixture was cooled in an ice bath and Hunig’s base (1.39 mL, 7.97 mmol, 2.25 eq) and EDCI (814 mg, 4.25 mmol, 1.2 eq) were added. After 20 minutes the ice bath was removed, and the reaction gradually became a light-brown solution. Radio-TLC reaction monitoring (silica gel GF; 98:2 ethyl acetate : methanol) indicated >98% (5) formation at 6 hours. A solution of water (3.5 mL), brine (3.5 mL) and 85% phosphoric acid (310 µL) was added dropwise to the reaction. Methyl t-butyl ether (5.4 mL) was then added, and the biphasic solution was vigorously stirred for 20 minutes. The resulting organic layer was isolated, washed with brine (5 mL), diluted with 1:1 methyl ethyl ketone : methyl t-butyl ether (10 mL), dried over anhydrous Na₂SO₄, filtered and concentrated under reduced pressure to afford a yellow-brown oil. The oil was dissolved in ethyl acetate (5 mL) and purified by flash column chromatography (silica gel: 98:2 ethyl acetate : methanol) to provide (5) as a white solid (ca. 186 mCi, 1503 mg, 3.18 mmol) in 90% yield.

[^14]C]N-((S)-1-(((S)-4-chloro-3-oxo-1-((S)-2-oxopyrrolidin-3-yl)butan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)-4-methoxy-1H-indole-2-carboxamide (6). 1-Methylpiperidine (3.87 mL, 31.8 mmol, 10 eq) was added to a round bottomed flask containing 1M t-butyl magnesium chloride in THF (63.6 mL, 63.6 mmol, 20 eq) and the resulting solution was cooled to approximately 10 °C using a water bath. A room temperature solution of (5) (ca. 186 mCi, 1503 mg, 3.18 mmol) and chloroacetic acid (751 mg, 7.95 mmol, 2.5 eq) in tetrahydrofuran (15 mL) was prepared and added dropwise to the Grignard solution over 30 minutes.
The cooling bath was removed, and the reaction was stirred at room temperature for approximately 18 hours under positive nitrogen pressure. Radio-TLC reaction analysis of a reaction aliquot partitioned between 25% aqueous citric acid and 2-methyltetrahydrofuran (silica gel GF; 98:2 ethyl acetate : methanol) indicated complete formation of (6). The reaction was then added dropwise over 30 minutes to an ice-cold solution of aqueous 25% citric acid (20 mL). The resulting biphasic mixture was warmed to room temperature and stirred for an additional 10 minutes. The organic layer was isolated, washed with aqueous 5% bicarbonate (30 mL) followed by brine (20 mL), dried over anhydrous Na₂SO₄ and filtered. The filtrate was immersed in a 60-70 ºC water bath and concentrated to approximately 15 mL using a slow stream of nitrogen gas. The resulting solution was cooled to 50 ºC, seeded with non-labeled (6) and the resulting suspension was stirred at this temperature for an additional 3 hours before it was allowed to cool to room temperature and stir for 3 hours more. The solids were collected by filtration, washed successively with 2-methyltetrahydrofuran, diethyl ether and pentane and dried under vacuum to afford (6) as a white solid (104 mCi, 936 mg, 1.91 mmol) in 60% yield. Specific activity of (6) was measured to be 54.3 mCi/mmol by gravimetric assay.

Radiodilution of (6). Methanol (0.5 mL) was added to (6) (ca. 18 mCi, 164 mg, 0.33 mmol) and unlabelled (6) (165 mg, 0.34 mmol) solids in a round bottomed flask. The resulting suspension was heated to 50 ºC and quickly became a solution. The solution was allowed to cool to room temperature and then further cooled in an ice-bath at which point solids precipitated. The suspension was held under refrigeration for ca. 16 hours and then filtered, rinsing the collected solids with ice-cold methanol (3 x 0.1 mL). The solids were dried under high vacuum to afford 181 mg of radiodiluted (6). This overall process was executed an additional time using (6) (ca. 16 mCi, 153 mg, 0.29 mmol) and unlabelled (6) (154 mg, 0.31 mmol) to afford 178 mg of radiodiluted (6). The two (6) products were thoroughly mixed to yield a single 352 mg sample of (6) with a specific activity of 24.0 mCi/mmol (49 μCi/mg), as measured by gravimetric assay.

[^14C]Lufotrelvir MEK Solvate. A round bottomed flask was charged with (6) (ca. 8.16 mCi, 166 mg, 0.34 mmol), methyl ethyl ketone (1.4 mL) and dimethylsulfoxide (0.25 mL). The resulting solution was treated with potassium di-t-butyl phosphate (116 mg, 0.47 mmol, 1.4 eq) and potassium iodide (12 mg, 0.07 mmol, 0.2 eq) and the resulting mixture was stirred at room temperature for two days. In process radio-HPLC analysis was performed on a reaction sample to confirm negligible unreacted (6) or iodo intermediate remained. Methyl t-butyl ether (1.7 mL) and water (1.7 mL) were added to the biphasic mixture, and it was vigorously stirred for 20 minutes. Two additional water washes were performed and then the organic layer was concentrated to dryness. The resulting oily residue was diluted with methyl ethyl ketone (10.9 mL) and concentrated to dryness under reduced pressure. This process was repeated three additional times at which point the residue appeared to be a solid. Oxalic acid (166 mg, 1.84 mmol, 5.4 eq) followed by methyl ethyl ketone (6.7 mL) was added to this solid to give a solution. The solution was concentrated to dryness under reduced pressure. Two additional cycles of methyl ethyl ketone (6.7 mL) addition and concentration were performed and then the solid residue was dissolved in methyl ethyl ketone (1.7 mL) and stirred at 60 ºC. After 2.5 hours the reaction was assayed by HPLC and deemed to be complete. The reaction was cooled in an ice-bath and the formed solids were collected by filtration, rinsed with ice-cold methyl ethyl ketone (4 x 0.5 mL) and dried under vacuum to afford [^14C]lufotrelvir MEK Solvate (ca. 2.2 mCi, 56 mg, 0.09 mmol) as a tan solid with a radiochemical purity of 97.8% (radio-HPLC) and a specific activity of 24.4 mCi/mmol (39.5 μCi/mg) as measured by gravimetric assay.
[^14C]Lufotrelvir IV Specific Activity. Radiodilution of[^14C]lufotrelvir MEK Solvate to the desired final specific activity was performed stepwise.

Methanol (22 mL) was added to[^14C]lufotrelvir MEK Solvate (ca. 1.57 mCi, 40 mg, 0.06 mmol) and lufotrelvir (505 mg, 0.89 mmol) solids in a round bottomed flask. The resulting suspension was heated at 55 °C for approximately 15 minutes. Additional methanol (6 mL) was added dropwise to the heated suspension to form a clear solution. The solution was then cooled and concentrated to dryness under reduced pressure at room temperature. The resulting solids were transferred to a filter funnel using methanol rinses (5 x 1.5 mL) and dried by filter suction followed by high vacuum to yield[^14C]lufotrelvir (ca. 1.06 mCi, 392 mg, 0.69 mmol) as a white solid with a specific activity of 2.70 μCi/mg (gravimetric assay).

A solution of[^14C]lufotrelvir (ca. 38 μCi, 14 mg, 0.02 mmol) in methanol (11 mL) was filtered into a 3 L Erlenmeyer flask and rinsed with additional methanol (3 x 2.2 mL). Lufotrelvir (45.98 g, 80.59 mmol) followed by methanol (1840 mL) was added to the flask and the resulting suspension was heated at 55 °C for approximately 15 minutes. Additional methanol (506 mL) was then added slowly to the heated suspension to fully dissolve remaining solids. The solution was then cooled and concentrated to dryness under reduced pressure at room temperature. The resulting solids were transferred to a filter funnel using methanol rinses (3 x 51 mL) and dried by filter suction followed by high vacuum to yield[^14C]lufotrelvir IV Specific Activity (ca. 32 μCi, 41.95 g, 73.53 mmol) as a white solid with a specific activity of 0.00077 μCi/mg (gravimetric assay).
SUPPLEMENTAL FIGURE 1. Mass Spectra for Lufotrelvir

[Diagram showing mass spectra with m/z values and relative abundance]
SUPPLEMENTAL FIGURE 2. Mass Spectra for PF-00835231

![Mass Spectra Image]

NL: 1.12E7
20201015-04a#484-492
RT: 8.23-8.27 AV: 4 F:
FTMS + p ESI Full ms
[100.00-800.00]

NL: 8.54E6
20201015-04a#483-490
RT: 8.23-8.26 AV: 4 F:
FTMS + c ESI Full ms2
473.00@cid35.00
[130.00-500.00]
SUPPLEMENTAL FIGURE 3. Mass Spectra for Epimer of PF-00835231
SUPPLEMENTAL FIGURE 4. Mass Spectra for Leucine

- Mass Spectra for Leucine with m/z values and relative abundance.
- The compound 
  \[ \text{H}_3\text{N}^+\text{CH}_2\text{CH}_2\text{COOH} \]

- Relative Abundance:
  - 132.1019
  - 182.0811
  - 146.1174
  - 116.0708
  - 137.0457
  - 160.1331
  - 191.0225
  - 175.0451
  - 123.0442
  - 104.1073
  - 87.1004
  - 117.9712
  - 92.1020
  - 78.0412
  - 140.8656
  - 60.7008

- Technical Details:
  - NL: 2.62E8
  - 20220504_P9803_04_WV16_005
  - #530-594 RT: 1.19-1.32 AV: 16
  - T: FTMS + p ESI Full ms
  - [100.0000-1500.0000]

- NL: 7.80E5
  - 20220504_P9803_04_WV16_005
  - #498-601 RT: 1.11-1.33 AV: 6
  - F: FTMS + p ESI d Full ms2
  - 132.1019@hcd35.00
  - [50.0000-125.0000]
SUPPLEMENTAL FIGURE 5. Mass Spectra for Metabolite M1
SUPPLEMENTAL FIGURE 6. Mass Spectra for Metabolite M6a

NL: 9.93E7
20220504_P9803_04_WV16_005#
4714-4753 RT: 10.73-10.81 AV:
10 T: FTMS + p ESI Full ms
[100.0000-1500.0000]

NL: 2.91E5
20220504_P9803_04_WV16_005#
4698-4752 RT: 10.71-10.79 AV:
3 F: FTMS + p ESI d Full ms2
475.2549@hcd35.00
[50.0000-505.0000]
SUPPLEMENTAL FIGURE 7. Mass Spectra for Metabolite 6b
SUPPLEMENTAL FIGURE 8. Mass Spectra for Metabolite M7
SUPPLEMENTAL FIGURE 9. Mass Spectra for Metabolite M8

NL: 1.88E6
20220323_P9803_04_WV07_005#16
29-1652 RT: 3.86-3.92 AV: 6 SB:
43 3.73-3.87 , 3.97-4.21 T: FTMS
+p ESI Full ms
[100.0000-1500.0000]

NL: 2.29E5
20220323_P9803_04_WV07_006#13
97-1423 RT: 3.89-3.94 AV: 3 F:
FTMS + p ESI Full ms2
291.1339@hcd35.00
[50.0000-315.0000]
SUPPLEMENTAL FIGURE 10. Mass Spectra for Metabolite M9

NL: 1.03E6
20201015-04c#408-411
RT: 7.13-7.14 AV: 2 SB: 2
7.03-7.07 F: FTMS + p
ESI Full ms
[100.00-800.00]

NL: 1.12E6
20201015-04c#406-411
RT: 7.10-7.13 AV: 3 F:
FTMS + c ESI Full ms2
192.00@cid35.00
[50.00-220.00]
SUPPLEMENTAL FIGURE 11. Mass Spectra for Metabolite M10

![Mass Spectra for Metabolite M10](image-url)

- NL: 2.11E6
- 20201129a-04b#333-346
- RT: 5.97-6.09 AV: 7 F:
  - FTMS + p ESI Full ms
  - [150.00-1000.00]

- NL: 4.15E5
- 20201129a-04b#333-344
- RT: 5.98-6.07 AV: 6 F:
  - FTMS + c ESI Full ms2
  - [100.00-500.00]
SUPPLEMENTAL FIGURE 12. Mass Spectra for Metabolite M11
SUPPLEMENTAL FIGURE 13. Mass Spectra for Metabolite M12
SUPPLEMENTAL FIGURE 14. Mass Spectra for Metabolite m/z 287

[Graph showing mass spectra with m/z values and relative abundance]
SUPPLEMENTAL FIGURE 15. Mass Spectra for Metabolites m/z 467a and 467b
SUPPLEMENTAL FIGURE 16. Mass Spectra for Metabolites m/z 487a and 487b

m/z 487 at ~4.8 min

m/z 487 at ~5.0 min
SUPPLEMENTAL FIGURE 17. Mass Spectra for Metabolite m/z 491a

NL: 1.65E7
20220504_P9803_04_WV16_005#21
49-2170 RT: 4.61-4.86 AV: 6 SB:
17 4.71-4.75 , 4.97-5.07 T: FTMS
+ p ESI Full ms
[100.0000-1500.0000]

NL: 6.02E5
20220610_P9803_04_WV18_002#17
39-1805 RT: 4.73-4.89 AV: 8 F:
FTMS + p ESI Full ms2
491.2497@hcd35.00
[50.0000-520.0000]
SUPPLEMENTAL FIGURE 18. Mass Spectra for Metabolites m/z 491b and 491c

m/z 491 at ~8.6 min

m/z 491 at ~9.4 min