Cytochrome P450 2B6 and UGT Enzymes-Mediated Clearance of Ciprofol (HSK3486) in Humans: The Role of Hepatic and Extrahepatic Metabolism

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**Abbreviations:**

BSA, bovine serum albumin; CYP, cytochrome P450; \( f_{\text{CL}} \), fraction of clearance; HLMs, human liver microsomes; HKMs, human kidney microsomes; HLS9s, human liver S9 fractions; HMBC, heteronuclear multiple bond correlation; IVIVE, *in vitro* to *in vivo* extrapolation; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LC-HRMS, liquid chromatography-high resolution mass spectrometry; NMR, nuclear magnetic resonance; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PBS, Phosphate-buffered saline; PAPS, adenosine 3’-phosphate 5’-phosphosulfate; UGT, uridine 5′-diphospho-glucuronosyltransferase; UDPGA, uridine 5′-diphosphoglucuronic acid; UPLC-UV/Q-TOF MS, ultra-high-performance liquid chromatography-ultraviolet-quadrupole time-of-flight mass spectrometry.
Abstract

Ciprofol (HSK3486) is a novel intravenous agent for general anesthesia. In humans, HSK3486 mainly undergoes glucuronidation to form M4 (fraction of clearance $[f_{CL}]$: 62.6%), followed by the formation of mono-hydroxylated metabolites that further undergo glucuronidation and sulfation to produce M5-1, M5-2, M5-3, and M3 (summed $f_{CL}$: 35.2%). However, the complete metabolic pathways of HSK3486 in humans remain unclear. In this study, by comparison with chemically synthesized reference standards, three mono-hydroxylated metabolites (M7-1, 4-hydroxylation with an unbound intrinsic clearance $[CL_{int,u}]$ of 2211 μL/min/mg; M7-2, ω-hydroxylation with a $CL_{int,u}$ of 600 μL/min/mg, and M7-3, (ω-1)-hydroxylation with a $CL_{int,u}$ of 78.4 μL/min/mg) were identified in human liver microsomes, and CYP2B6 primarily catalyzed their formation. In humans, M7-1 was shown to undergo glucuronidation at the 4-position and 1-position by multiple UDP-glucuronosyltransferases (UGTs) to produce M5-1 and M5-3, respectively, or was metabolized to M3 by cytosolic sulfotransferases. M7-2 was glucuronidated at the ω position by UGT1A9, 2B4, and 2B7 to form M5-2. UGT1A9 predominantly catalyzed the glucuronidation of HSK3486 (M4). The $CL_{int,u}$ values for M4 formation in human liver and kidney microsomes were 1028 and 3407 μL/min/mg, respectively. In vitro/in vivo extrapolation analysis suggested that renal glucuronidation contributed approximately 31.4% of the combined clearance. In addition to HSK3486 glucuronidation (M4), 4-hydroxylation (M7-1) was identified as another crucial oxidative metabolic pathway ($f_{CL}$: 34.5%). Further attention should be paid to the impact of CYP2B6- and UGT1A9-mediated drug interactions and gene polymorphisms on the exposure and efficacy of HSK3486.
Significance Statement

This research elucidates the major oxidative metabolic pathways of HSK3486 (the formation of three mono-hydroxylated metabolites: M7-1, M7-2, M7-3) as well as definitive structures and formation pathways of these mono-hydroxylated metabolites and their glucuronides or sulfate in humans. This research also identifies major metabolizing enzymes responsible for the glucuronidation (UGT1A9) and oxidation (CYP2B6) of HSK3486 and characterizes the mechanism of extrahepatic metabolism. The above information is helpful in guiding the safe use of HSK3486 in the clinic.
Introduction

General anesthetics are certain drugs that deprive patients of consciousness, pain, and physiological reflexes for therapeutic purposes. Currently, general anesthetics can be divided into three categories according to their relative potencies to reach different clinical endpoints: (1) ‘intravenous anesthetics’, such as propofol, etomidate, and barbiturates; (2) ‘volatile halogenated anesthetics’, such as halothane, enflurane, and isoflurane, and (3) ‘gaseous anesthetics’, such as nitrous oxide (N\textsubscript{2}O), xenon (Xe) and cyclopropane (Solt and Forman, 2007; Forman and Chin, 2008).

Propofol (Fig. 1A) is a classic anesthetic drug that exerts its effects by activating GAB\textsubscript{A} receptors (Vanlersberghe and Camu, 2008). Owing to favorable pharmacokinetics and pharmacodynamics, propofol has several advantages: rapid onset of action, fast awakening, complete functional recovery, and low incidence of postoperative vomiting (Dinis-Oliveira, 2018; Sahinovic et al., 2018). However, propofol is associated with several adverse reactions, including hypotension, respiratory depression, hypertriglyceridemia, and injection pain. Furthermore, the prolonged administration of high-dose propofol can cause propofol infusion syndrome (PRIS); this can cause death (Marik, 2004).

Ciprofol (code name HSK3486) (Fig. 1B) is an innovative GAB\textsubscript{A} agonist with a short duration of action. This compound was specifically designed to facilitate the initiation and continuity of general anesthesia and procedural sedation. (Qin et al., 2017; Liao et al., 2022). Unlike propofol, a symmetrical molecule, HSK3486 is a single enantiomer with an R-configuration chiral center. The introduction of a cyclopropyl group led to a slight increase in lipophilicity and a significant enhancement of anesthetic potency (Qin et al., 2017). The marketing authorization for HSK3486 was granted in China in 2020 following a priority review. Several
international studies have hitherto exhibited the clinical advantages of HSK3486 over propofol in terms of the lower doses required for anesthesia or sedation (0.2 - 0.6 mg/kg for HSK3486 versus 1.0 - 2.5 mg/kg for propofol) (Hu et al., 2021; Teng et al., 2021; Li et al., 2022; Luo et al., 2022).

A previous study investigated the pharmacokinetics, metabolism, and excretion of HSK3486 in six healthy participants following a single intravenous administration of 0.8 μCi/0.4 mg/kg Carbon-14 labeled HSK3486 (Bian et al., 2021). Analysis showed that HSK3486-1-O-β-glucuronide (M4, Fig. 1C) was the predominant circulating metabolite (79.3% of radioactive AUC), while HSK3486 constituted only 3.97%. The elimination of radioactivity through urine and feces was 84.6 and 2.65% in 240 h, respectively. The single major metabolite M4 in urine was identified (51.6% of dose). Additionally, three glucuronide conjugates of mono-hydroxylated HSK3486 (M5-1 [19.3% of dose], M5-2 [0.3% of dose], M5-3 [6.9% of dose]), and one sulfate conjugate of mono-hydroxylated HSK3486 (M3, [2.3% of dose]) were found in urine.

Based on the normalized data of these excretory metabolites (Supplementary Table 1), it was estimated that glucuronidation (M4) contributed 62.6% of the systemic clearance, while the summation of glucuronide and sulfate conjugates of mono-hydroxylated HSK3486 contributed approximately 35.2%. Notably, only one mono-hydroxylated metabolite (M7) was detected in the plasma and feces; theoretically, M7 produces at most two glucuronides in a manner that is inconsistent with the three glucuronides (M5-1–M5-3) present (Bian et al., 2021). In contrast, metabolite profiling of HSK3486 in human liver microsomes (HLMs) and human liver S9 fractions (HLS9s) showed the formation of three mono-hydroxylated metabolites (M7-1, M7-2, and M7-3) that were further converted to M5-1, M5-2, and M3 (Liao et al., 2022). However, the structures and formation pathways of these metabolites are
not well characterized. It is vital to understand which mono-hydroxylated metabolites are metabolized to the corresponding conjugated metabolites and identify the enzymes responsible for forming these mono-hydroxylated metabolites. Furthermore, the systemic clearance of HSK3486 in humans (39.9 ml/min/kg) (Bian et al., 2021) was higher than the normal hepatic blood flow (20.1 ml/min/kg) as described in (Davies and Morris, 1993). This discrepancy implies the potential involvement of extrahepatic organs in the metabolic processes of HSK3486. Unfortunately, the types and extent of extrahepatic metabolism have yet to be elucidated; this is not conducive to establishing reliable pharmacokinetic models for HSK3486 target-control infusion in different populations.

In this study, we comprehensively characterized structures of M7-1–M7-3 and M5-1–M5-3 formed in vitro and in vivo by chemically or biochemically synthesized standards. In addition, the formation pathways of human urinary metabolites were determined by profiling downstream metabolites of M7-1–M7-3 formed in HLS9s. As a result, the formation of M7-1 was confirmed as the single primary oxidative metabolic pathway of HSK3486 in humans. In vitro reaction phenotyping studies revealed that CYP2B6 and UGT1A9 mainly contributed to producing M7-1 and M4, respectively. Furthermore, we also evaluated the contribution of renal-mediated glucuronidation to HSK3486 metabolism.
Materials and Methods

Materials

HSK3486, HSK3486-1-O-β-glucuronide (M4), and five mono-hydroxylated HSK3486 candidate compounds (CV5-18-05-1 [M7-1], HE6-107-59-1 [M7-2], HE6-44-43-1, HE6-107-52-1 [M7-3], and HE6-44-29-1B) (Fig. 2B-2F) were generously offered by Haisco Pharmaceutical Group Co., Ltd. (Chengdu, China). CV5-18-05-1 is supplied as a single enantiomer, while the other four mono-hydroxylated compounds are racemates. HE6-107-59-1 (M7-2) is a mixture of two epimers (PH-HSC-008-0A-1 and PH-HSC-008-0C-1) that share the same R-configuration as HSK3486 on the cyclopropyl group side, but have opposite configurations (R/S) on the isopropyl group side. Phenacetin, acetaminophen, bupropion, hydroxybupropion, mephenytoin, 4’-hydroxymephenytoin, midazolam, 1’-hydroxymidazolam, testosterone, and 6β-hydroxy testosterone were from Toronto Research Chemicals (Toronto, Canada). Alamethicin was provided by Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). UDPGA and PAPS were acquired from Sigma-Aldrich (Saint Louis, MO). NADPH tetrasodium salt, fatty acid-free BSA, Niflumic acid, α-Napthoflavone, ticlopidine, N-benzylnervanol, ketoconazole, mefenamic acid, and propofol were obtained from Dalian Meilunbio Co., Ltd. (Dalian, China). Mixed-gender pooled 20-donor HLMs, cDNA-Expressed UDP-Glucuronosyl Transferase Enzymes, and Cytochrome P450 Enzymes (Supersomes) were acquired from Corning (Woburn, MA). The positive control data of these cDNA-Expressed Supersomes are included in Supplementary Table 2 and Table 3. Mixed-gender pooled 50-donor
HLS9s and 8-donor HKMs were obtained from XenoTech (Kansas City, KS).

**Metabolite Identification and Profiling in Healthy Volunteers**

Human plasma samples of six male participants were acquired from an open-label, two-stage clinical study (NCT05181007). Plasma samples at different time intervals were pooled in equal volumes to obtain a blank plasma sample, a 4 - 15 min pooled plasma sample, and a 30 min - 2 h pooled plasma sample, followed by extraction utilizing acetonitrile (3-fold volume). Given the volatility of HSK3486, a small portion of the supernatant from the extracted sample after centrifugation (18,800 g, 4°C, 10 min) was taken for direct UPLC-UV/Q-TOF MS detection. To improve the mass chromatogram peak response of the plasma metabolites, neglecting the loss of HSK3486. The leftover liquid was dried by employing N₂ at a temperature of 40°C and then mixed back together with acetonitrile-water (50/50). The concentrated plasma sample was subjected to analysis utilizing UPLC-UV/Q-TOF MS.

The urine samples of six male participants from a phase 1 study (NCT04145596) were pooled in proportion to their volume to obtain samples at 0 - 8 h, 8 - 24 h, and 24 - 48 h post-dose and extracted utilizing acetonitrile (equal volume). HSK3486 was not detectable in the urine samples; thus, the extracted urine supernatant was also concentrated before analysis.

**In Vitro Metabolism of HSK3486 and its Metabolites**

**Oxidative Metabolism of HSK3486 in HLMs.** A 100 μL incubation system comprising HLMs (0.5 mg/mL), HSK3486 (23 μM), and NADPH (1.0 mM) was used for the experiment. Each component was formulated with 100 mM of PBS (pH 7.4) containing 3.2 mM of MgCl₂. At the end of a 3-minute prewarming procedure (37°C)
in a thermo shaker incubator for 96-well plates, the buffer-microsome-substrate mixtures were supplemented with NADPH to commence the reaction.

**Glucuronidation and Sulfation of Mono-hydroxylated HSK3486 in HLMs and HLS9s.** For the *in vitro* glucuronidation reaction, the 100 μL incubation system consisted of HLMs (0.5 mg/mL), alamethicin (25 μg/mL), UDPGA (2.0 mM), and 23 μM of individual mono-hydroxylated HSK3486 compounds, which were CV5-18-05-1 (M7-1), HE6-107-52-1 (M7-3), and HE6-107-59-1 (M7-2) as well as two epimers of M7-2 (PH-HSC-008-0A-1/PH-HSC-008-0C-1). All components were formulated with 100 mM Tris-HCl (pH 7.4) containing 10 mM of MgCl₂. Additionally, HLMs were activated utilizing alamethicin on ice for 15 min to form pores. The incubation system for the *in vitro* sulfation reaction comprised HLS9s (2.0 mg/mL), PAPS (1.0 mM), and 23 μM of individual mono-hydroxylated HSK3486 compounds. The commencement of reactions was achieved by incorporating UDPGA or PAPS into the incubation system at the end of a 3-minute prewarming procedure at 37°C.

**Metabolism of Mono-hydroxylated HSK3486 in HLS9s.** The incubation system for this part of the study was similar to the *in vitro* glucuronidation reaction described above. HLS9s were formulated at a final concentration of 2.0 mg/mL, and the three mono-hydroxylated compounds (CV5-18-05-1 [M7-1]/HE6-107-59-1 [M7-2]/HE6-107-52-1 [M7-3]) were added individually as reaction substrates. UDPGA and NADPH were also added to ensure that both the oxidation and glucuronidation pathways could occur.

All incubated samples (n=2) were halted with an equivalent volume of chilled acetonitrile after 60 min; proteins were removed by centrifugation (18,800 g, 4°C, 5 min), and the supernatant was analyzed utilizing UPLC-UV/Q-TOF MS. The
metabolites formed in vitro were compared with the reference standards as well as the metabolites detected in humans.

**Biosynthesis of M5-1 and M5-2 and Structural Elucidation by NMR.** M5-1 and M5-2 were prepared using CV5-18-05-1 (M7-1) and PH-HSC-008-0A-1 (one enantiomer of M7-2) as substrates, respectively. The scale-up incubation system (100 mL) consisted of 1.0 mg/mL of HLMs, 2.0 mM of UDPGA, 25 µg/mL of alamethicin, and 500 µM of CV5-18-05-1 or 227 µM of PH-HSC-008-0A-1. The prepared metabolites were then extracted with acetonitrile and purified by semi-preparative liquid chromatography. Subsequently, the collected metabolite fractions were dried with a rotary evaporator and dissolved in deuterated methanol for ¹H and ¹³C NMR analysis by AVANCE III NMR spectrometers (Bruker GmbH, Mannheim, Germany).

**UPLC-UV/Q-TOF MS Analysis of HSK3486 Metabolites.**

The processed samples from in vivo or in vitro metabolism study were analyzed utilizing a liquid chromatography-high resolution mass spectrometry (LC-HRMS) platform equipped with Acquity UPLC-UV and Synapt G2-Si Q-TOF (Waters, USA) in negative ion mode. A detailed summary of the operating parameters is provided in the Supplementary Method 1.

**CYP Reaction Phenotyping of HSK3486**

**Kinetic Analysis of HSK3486 Oxidative Metabolism in HLMs.** HSK3486 (1 µM - 500 µM) and HLMs (0.1 mg/mL) were formulated in PBS with or without 2% fatty acid-free BSA. Oxidative metabolism was commenced by introducing NADPH (1.0 mM) after prewarming (37°C, 3min). Chilled acetonitrile was utilized to halt the reactions after 10 min. Control samples containing blank organic solvents were also
prepared to determine background values. All three mono-hydroxylated metabolites (M7-1–M7-3) were formed in the linear range. Subsequently, these metabolites were quantified utilizing an LC-MS/MS approach.

**Metabolism of HSK3486 in Human Recombinant CYPs.** The qualitative screening of CYPs engaged in forming the three mono-hydroxylated metabolites (M7-1–M7-3) was conducted with 10 Human CYP Supersomes (1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5). HSK3486 (1 μM), NADPH (1.0 mM), and each CYP Supersome (15 pmol/mL) were co-incubated for a duration of 15 min. Samples were extracted and subsequently subjected to analysis utilizing LC-MS/MS.

**CYP Inhibition Analysis.** Based on recombinant CYP enzyme screening, α-naphthoflavone (2 μM, CYP1A2), ticlopidine (3 μM, CYP2B6), N-benzylnirvanol (3 μM, CYP2C19) and ketoconazole (2 μM, CYP3A) were added to HLMs (0.1 mg/mL) individually to quantitatively investigate the contribution of particular CYP isoforms to the formation of the three mono-hydroxylated metabolites (M7-1–M7-3). Ticlopidine is a mechanism-based inhibitor, necessitating a pre-incubation period of 15 min with microsomes and NADPH. This was followed by adding HSK3486 (1 μM) to commence the reaction for 10 min. The chemical inhibitor in the control group was substituted with a blank buffer. The mono-hydroxylated metabolites were subsequently quantified utilizing LC-MS/MS. The effect of each chemical inhibitor on the metabolic rates of different CYP probe substrates was also examined to assess the potential off-target inhibitory effect.

**UGT Reaction Phenotyping of HSK3486**

**Kinetic Analysis of HSK3486 Glucuronidation Metabolism in HLMs and HKMs.** The entire incubation mixture (100 μL) was prepared in Tris-HCl buffer with
or without 2% fatty-acid-free BSA, including HLMs (0.1 mg/mL) or HKMs (0.05 mg/mL) and HSK3486 (2 µM - 800 µM). Microsomal protein was activated utilizing alamethicin (25 µg/mL) for 15 min, succeeded by a 3-minute prewarming process at 37°C. Glucuronidation metabolism was commenced with UDPGA (2 mM) and halted with chilled acetonitrile after 10 min. M4 was quantified utilizing an LC-MS/MS approach alongside calibration standards.

Metabolism of HSK3486 and Mono-hydroxylated Metabolites in Human Recombinant UGTs. 12 Human UGT Supersomes (1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17, final concentration 0.1 mg/mL) were incubated with alamethicin, UDPGA and 1 µM of the individual substrate (HSK3486/M7-1/M7-2) for 25 min. Samples were extracted and subsequently subjected to analysis utilizing LC-MS/MS.

UGT Inhibition Analysis. The composition of the incubation mixture closely resembled that employed for UGT enzyme kinetic analysis, as described above, but without fatty acid-free BSA. Niflumic acid (5.0 µM) was used as a UGT1A9 inhibitor (Miners et al., 2011), and the concentration of individual substrate (HSK3486/M7-1/M7-2) was set at 1 µM. HLMs were used at 0.1 mg/mL. In the control group, niflumic acid was replaced with Tris-HCl buffer containing an equal proportion of organic solvent. Reactions were commenced by introducing UDPGA and ended 25 minutes later with chilled acetonitrile. Tris-HCl buffer and niflumic acid were added separately to the inhibitor and control groups to eliminate matrix differences. Subsequently, samples were centrifuged, and analyzed utilizing LC-MS/MS.

Determining the Unbound Fraction of HSK3486 from In Vitro Incubation Systems by Ultracentrifugation
HSK3486 (5 μM or 25 μM) was prepared in HLMs or HKMs with or without 2% BSA, as described above; UDPGA or NADPH was replaced with a blank buffer. Microsomal samples containing HSK3486 were divided into two portions. One portion was mixed with an equal volume of pre-centrifuged blank supernatant to determine the total drug concentration (C₀). The other portion was packed into an OptiSeal polypropylene tube (Beckman, CA, USA), sealed with a rubber stopper to ensure the absence of air bubbles, and equilibrated for 15 min at 37°C. Samples were then centrifuged utilizing an Optima MAX-TL benchtop ultracentrifuge (Beckman, CA, USA) at 37°C and 657000 g for 4 h. Subsequently, the supernatant was aspirated and combined with an equal amount of blank microsomes to determine the concentration of unbound drug (Cᵢ) by LC-MS/MS. The unbound fraction of HSK3486 was calculated by the following formula: fᵤ = Cᵢ/C₀ × 100%. Additional samples (with the same concentration) were prepared in polypropylene tubes and sealed to investigate the stability of HSK3486 over 5 h.

**Determination of HSK3486 and its Metabolites by LC-MS/MS**

The metabolites of HSK3486 from *in vitro* reaction phenotyping experiments and the parent drug in unbound fraction assay were detected utilizing a sensitive LC-MS/MS approach as described in Supplementary Method 2.

**Data Analysis**

**Enzyme Kinetic Data Analysis and *In Vitro*/*In Vivo* Extrapolation**

The kinetic parameters of a particular metabolic pathway were obtained using GraphPad Prism 9 software. The one-enzyme Michaelis-Menten model (eq. 1), the two-enzyme Michaelis-Menten model (eq. 2), and the allosteric sigmoidal model (eq. 3) were used for non-linear curve fitting. The goodness-of-fit for each model applied
was determined by $R^2$ value, the F-test ($P < 0.05$), and Akaike’s information criterion value.

\[
V = \frac{V_{\text{max}} \times S}{K_m \times S} \quad (1)
\]

\[
V = \frac{V_{\text{maxHi}} \times S}{K_{mHi} \times S} + \frac{V_{\text{maxLo}} \times S}{K_{mLo} \times S} \quad (2)
\]

\[
V = \frac{V_{\text{max}} \times S^n}{S_{50}^n + S^n} \quad (3)
\]

In eq.2, the subscripts “Hi” and “Lo” stand for the “high-affinity/low-capacity” enzyme and the “low-affinity/high-capacity” enzyme, respectively.

\[
CL_{\text{int,u}} = \frac{V_{\text{max}}}{K_m} \times f_u \quad (4)
\]

\[
CL_{\text{int,u}} = \frac{V_{\text{max}}}{S_{50}} \times \frac{n - 1}{n \times (n - 1)^{1/n}} \times f_u \quad (5)
\]

The in vitro unbound intrinsic clearance ($CL_{\text{int,u}}$) was calculated by using eq. 4 and eq. 5, respectively (Brian Houston, 1994; Houston and Kenworthy, 2000) and further extrapolated to the whole organ level ($CL_{\text{int,sc,u}}$) via liver and kidney scaling factors according to eq. 6. The microsome scaling factors (mg microsome protein/g organ) were 32 and 13.6 for the liver and kidney in humans, respectively; the reported organ weights (g/kg) were 25.7 (liver) and 4.4 (kidney), respectively (Davies and Morris, 1993; Barter et al., 2007; Scotcher et al., 2016).

\[
CL_{\text{int,sc,u}} = CL_{\text{int,u}} \times \frac{mg \text{ Microsome Protein}}{g \text{ Organ}} \times \frac{g \text{ Organ}}{kg \text{ Body Weight}} \times \frac{mL}{1000} \quad (6)
\]

The percentage of a single pathway to the general clearance was performed by dividing the $CL_{\text{int,sc,u}}$ value of an individual metabolic pathway by the sum of the $CL_{\text{int,sc,u}}$ value of all metabolic pathways (eq. 7).
In vitro blood clearance at the organ level was estimated employing the well-stirred model (eq. 8) (Brian Houston, 1994) in which \( Q \) represents organ blood flow (21 and 18 mL/min/kg for liver and kidney, respectively), and \( f_{ub} \) represents the unbound drug ratio in whole blood (the blood-to-plasma ratio for HSK3486 was 0.59, while the unbound fraction for HSK3486 in plasma was 0.05) (Bian et al., 2021; Liao et al., 2022). \( CL_{int,sc,u} \) represents the unbound intrinsic clearance of the organ.

\[
CL_b = Q \times \frac{f_{ub} \times CL_{int,sc,u}}{Q + f_{ub} \times CL_{int,sc,u}}
\] (8)

Correction of the Off-target Inhibition Effect of Chemical Inhibitors with the Application of a Coefficient Matrix

The metabolite formation rate (\( k \)) was measured with and without the addition of chemical inhibitors to obtain the percentage inhibition (eq. 9). The off-target inhibition data for chemical inhibitors, along with uncorrected inhibition values, were substituted into eq. 10 to determine corrected inhibition values (Njuguna et al., 2016).

\[
\%\text{Inhibition}_X = \left( 1 - \frac{\text{Inhibitor } k}{\text{No Inhibitor } k} \right) \times 100
\] (9)

\[
\begin{bmatrix}
I_{aA} & I_{aB} & I_{aC} \\
I_{bA} & I_{bB} & I_{bC} \\
I_{cA} & I_{cB} & I_{cC}
\end{bmatrix}
\times
\begin{bmatrix}
\text{Inhibition}_{X,CYP A, corrected} \\
\text{Inhibition}_{X,CYP B, corrected} \\
\text{Inhibition}_{X,CYP C, corrected}
\end{bmatrix}
= 
\begin{bmatrix}
\text{Inhibition}_{X,CYP A} \\
\text{Inhibition}_{X,CYP B} \\
\text{Inhibition}_{X,CYP C}
\end{bmatrix}
\] (10)

\%\text{Inhibition}_X \) represents the contribution of one CYP to a single metabolic pathway \( X \) while the \( 3 \times 3 \) coefficient matrix represents the off-target inhibition effect of chemical inhibitors (as assessed by CYP marker reactions). \( I_{aA} \) represents the percentage inhibition of inhibitor \( a \) on enzyme \( A \). \( \text{Inhibition}_{X,CYP A} \) represents the uncorrected contribution of enzyme \( A \) to metabolic pathway \( X \), as presented by inhibition value. \( \text{Inhibition}_{X,CYP A, corrected} \) represents the corrected contribution value of
enzyme $A$. 
Results

Identification of HSK3486 Metabolites in Human Plasma and Urine

Based on radioactive metabolite profiles reported in previous human radiolabeled mass balance study (Bian et al., 2021), we re-optimized our chromatographic conditions on a UPLC system to ensure separation of the major metabolite isomers within 15 min. Supplementary Fig. 1 demonstrates the representative metabolic profiles of HSK3486 in humans. The LC-HRMS data (relative retention times, accurate mass, MS/MS spectra, and the relative intensity of mass chromatogram peaks) of the identified metabolites in the current study were compared with those previously published (Bian et al., 2021). The code names of major metabolites identified in the present investigation were in accordance with those previously published. The newly identified metabolites were numbered in order of their mass-to-charge ratio and retention time. The trace metabolites M10-1, M10-2, M8-1, M8-2, and M9 reported previously (Bian et al., 2021) were renamed in the present study as M11-1, M11-2, M12-1, M12-2, and M13, respectively.

Plasma Analysis. A total of seven or thirteen metabolites were found in unconcentrated or concentrated plasma from healthy subjects (Supplementary Fig. 1A and 1B). Although significant depletion of the parent drug was observed after concentration (probably due to volatilization), metabolites with increased polarity exhibited a higher MS response. The characterized metabolites in human plasma included M1 (methyl to carboxylation); M2-1–M2-3 (methyl to carboxylation and mono-hydroxylation); M3 (mono-hydroxylation and sulfation); M4 (glucuronidation); M5-1–M5-3 (mono-hydroxylation and glucuronidation); M7 (mono-hydroxylation); M6-1 (di-hydroxylation and glucuronidation), and M11-1 and M11-2 (mono-
hydroxylation, dehydrogenation and glucuronidation). The six plasma metabolites (M3, M4, M5-1–M5-3, and M7) reported in the prior investigation (Bian et al., 2021) were all detected in the current study. However, we did not detect M2-4, the carboxylic acid metabolite. In addition, seven trace metabolites (M1, M2-1~M2-3, M6-1, M11-1, and M11-2) were newly identified in the present study. M4 still exhibited the highest abundance based on the MS peak area.

**Urine Analysis.** A total of twenty metabolites were detected in urine (Supplementary Fig. 1C and 1D). In addition to the previously reported M2-1~M2-3, M3, M4, M5-1–M5-3, M6-1, M6-3~M6-5, M11-1, M11-2, M12-1 and M12-2 (methyl to carboxylation, mono-hydroxylation, and glucuronidation), M13 (tri-hydroxylation, and glucuronidation) (Bian et al., 2021), we identified three new metabolites (M6-6~M6-8) in the current study. M4 and M5-1 still exhibited the highest abundance based on the UV peak area.

**Characterization of HSK3486 Metabolites in HLMs and Structure Confirmation of M7-1, M7-2, and M7-3**

To determine the exact structures of M5-1–M5-3 and M3, it was first necessary to characterize their aglycons. Metabolite profiling of HSK3486 in HLMs fortified with NADPH revealed the formation of multiple oxidative metabolites (Fig. 2A), including three mono-hydroxylated metabolites (M7-1–M7-3; m/z 219.1391), two mono-hydroxylated and dehydrogenated metabolites (M10-1 and M10-2; m/z 217.1234), two di-hydroxylated metabolites (M8-1 and M8-2; m/z 235.1340), and one tri-hydroxylated metabolite (M9; m/z 251.1289). Only the UV signal of M7-1 was detected, thus implying that M7-1 was the most abundant metabolite during HLM incubation.
Next, we compared LC-HRMS data from the five chemically synthesized mono-hydroxylated HSK3486 candidates (CV5-18-05-1, HE6-107-59-1, HE6-44-43-1, HE6-107-52-1, and HE6-44-29-1B) (Fig. 2B-2I) and confirmed that M7-1 was CV5-18-05-1 in which the 4-position of the phenol had been hydroxylated. In addition, M7-2 was confirmed as HE6-107-59-1, in which the ω-position of the isopropyl group had been hydroxylated, and M7-3 was confirmed as HE6-107-52-1, in which the (ω-1)-position of the isopropyl group had been hydroxylated. The mono-hydroxylated metabolite detected in human plasma was finally determined as M7-3 (Data not shown). Notably, we were unable to separate HE6-107-59-1 (M7-2) and HE6-44-43-1, or HE6-107-52-1 (M7-3) and HE6-44-29-1B, on a regular C18 column. Fortunately, their MS/MS spectra were rather different: HE6-44-43-1 could cleave to lose the cyclopropyl group (m/z 148) when compared to HE6-107-59-1 (Fig. 2C and Fig. 2D). The isopropyl group loss occurred within HE6-107-52-1 (m/z 161) while the cyclopropyl ethyl group loss within HE6-44-29-1B (m/z 135), as depicted in Fig. 2E and Fig. 2F. Hydroxylation of the alkyl side chain of HSK3486 allowed the loss of the corresponding side chain during collision-induced dissociation, thus helping us to distinguish these isomers.

Structure Elucidation of M5-1, M5-2, M5-3 and M3

Analysis of M5-1–M5-3 by UPLC/Q-TOF MS. The elution of M5-3 and M5-1 (3.06 min and 3.54 min, m/z 395.1711) occurred more rapidly than that of M5-2 (4.33 min, m/z 395.1711) (Fig. 3A). Both M5-1 and M5-3 generated the mono-hydroxylated HSK3486 ion at m/z 219.139, as well as glucuronide acid-related ions at m/z 175.0248 (C₆H₇O₆⁻) and m/z 113.0244 (C₅H₅O₃⁻) (Fig. 3E and 3F). M5-2 (Fig. 3G) revealed a high abundance of intact glucuronic acid ion (m/z 193.0354; C₆H₇O₇⁻), while the
aglycone ion at \(m/z\) 219.1391 was almost undetectable. The structures of these isomers could not be characterized based on the chromatographic and HRMS data alone.

**In vitro** Glucuronidation and Sulfation of Mono-hydroxylated HSK3486. CV5-18-05-1 (M7-1), HE6-107-59-1 (M7-2), and HE6-107-52-1(M7-3) were individually incubated in HLMs fortified with UDPGA. The glucuronides formed from the three mono-hydroxylated metabolites were then compared with the *in vivo* metabolites. The two glucuronides formed by CV5-18-05-1 (M7-1) shared the same retention times and MS/MS spectra as M5-1 and M5-3, respectively (Fig. 3B). Similarly, the glucuronide formed by HE6-107-59-1 (M7-2) was determined to be M5-2 (Fig. 3C). Therefore, the aglycone of both M5-1 and M5-3 was identified as M7-1 while the aglycone of M5-2 was identified as M7-2. One sulfate conjugate generated by CV5-18-05-1 (M7-1) in the HLS9s fortified with PAPS shared the same LC-HRMS data as M3 (Supplementary Fig. 2). Thus, M7-1 was confirmed to be the primary metabolite that forms M3.

**Structural Characterization of M5-1 and M5-2 by NMR.** M5-1 and M5-2 were isolated and purified from scaled-up *in vitro* incubation systems for NMR analysis. The \(^{13}\text{C}-^{1}\text{H}\) HMBC spectra of M5-1 and M5-2 are shown in Supplementary Fig. 3. For M5-1, the 15-CH proton correlated with the 4-C carbon of the phenol, thus demonstrating the attachment of glucuronide acid to the 4-position phenolic hydroxyl group of M7-1 (Supplementary Fig. 3A). For M5-2, the protons and carbons of 15-CH and 14-CH\(_2\) correlated with each other (Supplementary Fig. 3B), thus suggesting the attachment of glucuronide acid to the alcoholic hydroxyl group of the \(\omega\)-position of the isopropyl group of M7-2. The complete NMR spectra of M5-1 and M5-2 are
summarized in the Supplemental Data A and B.

**Characterization of Metabolites of Mono-hydroxylated HSK3486 in HLS9s**

To fully characterize the formation pathways of the HSK3486 metabolites detected in humans, CV5-18-05-1 (M7-1), HE6-107-59-1 (M7-2), and HE6-107-52-1 (M7-3) were incubated separately with HLS9s fortified with NADPH and UDPGA to generate their metabolites; these were then compared with the metabolites detected in humans.

As illustrated in Supplementary Fig. 5, eight metabolites were found in HLS9s incubated with M7-1, including M5-1, M5-3, M6-1, M8-1, M8-2, M9, M10-1, and M11-1. Ten metabolites were detected in the HLS9s incubated with M7-2, including M1, M2-1~M2-3, M5-2, M6-1, M8-1, M9, M10-2 and M11-2. Three metabolites were detected in the HLS9s incubated with M7-3, including M6-2, M8-2, and M9. The downstream metabolites of these mono-hydroxylated metabolites in HLS9s shared some crossover. Both M7-1 and M7-2 could be further oxidized to a di-hydroxylated metabolite M8-1 (Supplementary Fig. 5A and 5B), while both M7-1 and M7-3 could produce another di-hydroxylated metabolite M8-2 (Supplementary Fig. 5A and 5C). M8-1 was hypothesized as the ω-hydroxy M7-1, while M8-2 was hypothesized to be the (ω-1)-hydroxy M7-1 (Fig. 4). The alcoholic glucuronide M6-1 (di-hydroxylation and glucuronidation) was detected both in M7-1 and M7-2 incubations, thus suggesting that its aglycone was M8-1. All three mono-hydroxylated metabolites were able to form the same tri-hydroxylated metabolite M9. We presumed that the structure of M9 was a combination of the three mono-hydroxylated metabolites (Fig. 4). The production of four carboxylic acid metabolites (M1, M2-1~M2-3) was only observed in the M7-2 incubation system (Supplementary Fig. 5B). M1 was hypothesized to represent the ω-carboxylation of HSK3486 (Fig. 4).
Furthermore, M10-1 and M10-2 (mono-hydroxylation and dehydrogenation), and their glucuronides (M11-1 and M11-2), could be generated by M7-1 and M7-2, respectively. The other trace metabolites (M6-3-M6-5, M12-1, M12-2, and M13; <1% of the dose excreted) identified in vivo were not detected in vitro.

**In Vitro CYP Phenotyping of HSK3486**

CYP screening results showed that substantial M7-1 was observed in CYP1A2, CYP2B6, and CYP3A4 incubations (Fig. 5A). Two CYPs (2B6 and 2C19) could catalyze the production of M7-2 (Fig. 5B). In addition, three CYPs (1A2, 2B6, and C19) revealed catalytic activities in the production of M7-3 (Fig. 5C).

The quantitative contribution of each CYP isoform producing these mono-hydroxylated metabolites was assessed by correcting the inhibition data with the coefficient matrix method (Njuguna et al., 2016) (Table 1). The off-target inhibitory effect of chemical inhibitors on CYP isoforms is given in Supplementary Table 4. The contribution of CYP2B6 to M7-1 formation was 95.2%; however, the contributions of CYP1A2 and CYP3A4 were negligible. CYP2B6 was mainly responsible for M7-2 formation; the relative contribution of CYP2C19 was negligible. For M7-3, the relative contributions of CYP1A2 and CYP2B6 were 30.7% and 69.3%, respectively.

**In Vitro UGT Phenotyping of HSK3486 and Mono-hydroxylated Metabolites**

Of the 12 screened UGT enzymes (Fig. 6A), UGT1A9 exhibited the most prominent activity in catalyzing the glucuronidation of HSK3486 to form M4. The relative catalytic activity of UGT1A7 was only 2.1% of that exhibited by UGT1A9, respectively. As with M4, UGT1A7, and UGT1A9 were also able to catalyze the formation of M5-3 from M7-1 (Fig. 6C), with UGT1A9 exhibiting the highest activity. UGT1A9 and multiple UGT 2Bs (2B4, 2B7, 2B15, and 2B17) were involved.
in the formation of M5-1 from M7-1 (Fig. 6B) while UGT1A9, UGT2B4, and UGT2B7 were able to catalyze M7-2 to form M5-2 (Fig. 6D). Niflumic acid (UGT1A9 inhibitor) significantly inhibited the production of M4 and M5-3 in HLMs (Fig. 6E), but had little effect on M5-1 and M5-2 (Fig. 6F).

**Enzyme Kinetics and the In Vitro Clearance of HSK3486 Metabolism in HLMs and HKMs**

The substrate saturation curves for M7-1 (Fig. 7A) and M7-3 (Fig. 7C) formation in HLMs with or without 2% BSA were both fitted with the two-enzyme Michaelis-Menten model (Table 2); however, M7-2 formation (Fig. 7B) conformed to the one-enzyme model well (Table 2). In the presence of 2% BSA, the $K_{mHi}$ or $K_{mLo}$ as well as $V_{maxHi}$ or $V_{maxLo}$ were 4.74 or 107 μM as well as 1090 or 1309 pmol/min/mg for M7-1, and 4.08 or 67.1 μM as well as 33.1 or 32.0 pmol/min/mg for M7-3, respectively. The $K_m$ and $V_{max}$ were 6.55 μM and 431 pmol/min/mg for M7-2. Analysis showed that M7-1 possessed the greatest CL_{int,u} value (2211 μL/min/mg); this was followed by M7-2 (600 μL/min/mg) and M7-3 (78.4 μL/min/mg). By comparison with the outcomes derived from incubations lacking 2% BSA, the inclusion of BSA increased the CL_{int,u} value of M7-1, M7-2, and M7-3 by 2.8, 5.4, and 8.3-fold, respectively. CL_{int,u} values were further scaled to the whole liver level with hepatic physiological parameters to obtain CL_{int,sc,u} values for these mono-hydroxylated metabolites. The sum of the three oxidative metabolic pathways yielded total CYP CL_{int,sc,u} values of 757 mL/min/kg in the absence of 2% BSA and 2376 mL/min/kg in the presence of 2% BSA, thus representing the total unbound intrinsic clearance of HSK3486 via oxidative metabolism by hepatic CYP enzymes.

The substrate saturation curves for M4 formation in HLMs and HKMs without incorporating BSA were consistent with the allosteric sigmoidal model (Fig. 8). The
S₅₀ₐ values for M₄ in HLMs and HKMs were 14.9 and 17.9 μM, respectively. The 
Vₘₐₓ value for M₄ in HKMs (4470 pmol/min/mg) was approximately three-fold 
higher than that in HLMs (1369 pmol/min/mg) (Table 3). The UGT CLₐ values for 
M₄ in HLMs and HKMs were 52.8 and 141 μL/min/mg, respectively. Hepatic and 
renal UGT CLₛ values were 43.4 and 8.41 mL/min/kg, respectively.

When 2% BSA was incorporated, the kinetic profiles of M₄ formation in HLMs 
and HKMs changed to the one-enzyme Michaelis-Menten model (Fig. 8 and Table 3). 
The S₅₀ₐ values of M₄ in HLMs and HKMs decreased to 1.96 and 1.89 μM while the 
Vₘₐₓ values increased to 2012 and 6442 pmol/min/mg, respectively. The UGT CLₐ values for 
M₄ in HLMs and HKMs were 1028 and 3407 μL/min/mg, respectively; 
these were 20-fold and 24-fold higher than in systems without 2% BSA. The scaled 
hepatic and renal UGT CLₛ values were 845 and 204 mL/min/kg, respectively.

The sum of the hepatic UGT CLₛ value (2% BSA) and the hepatic CYP 
CLₛ value (2% BSA) yielded a total hepatic CLₛ value of 3221 mL/min/kg. 
According to eq. 8, the liver blood clearance (CLₗ,ₗ) and the renal blood clearance 
(CLₗ,ₗ) were estimated to be 19.3 and 8.81 mL/min/kg, respectively. The combined 
clearance of the two organs was 28.1 mL/min/kg. Therefore, liver and kidney 
clearance accounted for 0.69 and 0.31, respectively (Table 4).
Discussion

In this study, a total of thirteen plasma metabolites and twenty urine metabolites were identified in volunteers received HSK3486 injection (Supplementary Fig. 1). Three glucuronide conjugates of mono-hydroxylated HSK3486 (M5-1–M5-3) and one sulfate conjugate of mono-hydroxylated HSK3486 (M3) were detected both in the current study and the previously published human radiolabeled mass balance study (Bian et al., 2021); these were considered to be the most important metabolites second only to the HSK3486-1-O-β-glucuronide (M4). To elucidate the structures of these secondary conjugated metabolites, we first evaluated the regioselective oxidation of HSK3486.

In vitro metabolite profiling experiments (Fig. 2A) revealed that HSK3486 was able to undergo 4-hydroxylation to produce M7-1 (CV5-18-05-1, Fig. 2B), ω-hydroxylation to produce M7-2 (HE6-107-59-1, Fig. 2C), and (ω-1)-hydroxylation to produce M7-3 (HE6-107-52-1, Fig. 2E) in HLMs when fortified with NADPH. M7-3 was further determined to be the mono-hydroxylated metabolite found in human plasma. Enzymatic kinetic studies conducted in HLMs with or without 2% BSA further revealed that M7-1 exhibited the highest unbound intrinsic clearance (2211 or 800 mL/min/kg); this was followed by M7-2 (600 or 112 mL/min/kg) and M7-3 (78.4 or 9.44 mL/min/kg) (Table 2). Metabolites in which the cyclopropyl ethyl side chain was hydroxylated (HE6-44-43-1 or HE6-44-29-1B) were not detected in HLMs. These results indicated that HSK3486 was more inclined to undergo hydroxylation at the para-position of phenol, followed by the isopropyl side. This regional selectivity may be due to the rich electron density at the para position of the phenol group and the hindered steric effect of the cyclopropyl group.

M7-1 was able to undergo further glucuronidation to form M5-1 and M5-3 in
HLMs (Fig. 3B) or sulfation to produce M3 in HLS9s (Supplementary Fig. 2). M7-2 was able to undergo glucuronidation to form M5-2 (Fig. 3C). Although a glucuronide was produced by M7-3 in HLMs when supplemented with UDPGA alone (Fig. 3D), this metabolite was not detected in humans. This may be due to the low amount of M7-3 formed \textit{in vivo} and its preference for oxidative pathways when compared with glucuronidation. Two-dimensional NMR analysis of the glucuronide reference standards prepared by biochemical synthesis revealed that the glucuronidation site of M5-1 was the 4-position phenolic hydroxyl group of M7-1 (Supplementary Fig. 3A) while the glucuronidation site of M5-2 was the ω-position alcoholic hydroxyl group of M7-2 (Supplementary Fig. 3B). Although M5-3 was not analyzed by NMR due to its low yield \textit{in vitro}, its glucuronidation site could only be the 1-position phenolic hydroxyl group of M7-1. Theoretically, M7-2 (HE6-107-59-1) included two epimers (PH-HSC-008-0A-1 and PH-HSC-008-0C-1); this was because both isopropyl methyl groups of HSK3486 could be hydroxylated. However, the separation of the two epimers could not be achieved in the present study. Similarly, M5-2 formed \textit{in vitro} or \textit{in vivo} also included a pair of epimers; furthermore, the separation of the two epimers (PH-HSC-008-0A-1 glucuronide and PH-HSC-008-0C-1 glucuronide) in human urine and HLMs (Supplementary Fig. 4) was achieved with a modified UPLC gradient method. M5-2 was an alcoholic glucuronide that was able to produce the intact glucuronide ion (m/z 193.0354) in negative mode (Fig. 3G); this was quite different from the mass fragment pattern of phenolic glucuronides such as M4, M5-1, and M5-3 (Fig. 3E and 3F). Thus, the characteristic fragment ions of glucuronide acid moiety could help to distinguish different types of O-glucuronides.

The metabolic profiles of the mono-hydroxylated HSK3486 (M7-1–M7-3) in HLS9s fortified with NADPH and UDPGA were analyzed to elucidate the formation
pathways of the remaining metabolites in humans. The major metabolites (M5-1–M5-3) and most of the minor metabolites detected in humans could be formed from the three mono-hydroxylated metabolites (Supplementary Fig. 5). We hypothesized that M7-1 and M7-2 were rapidly converted to their conjugated metabolites upon formation; this could explain why M7-1 and M7-2 were not detected in vivo (Supplementary Fig. 1). Based on these results, more definite metabolites’ structures and biotransformation pathways of HSK3486 are proposed in Fig. 4. M7-1 (the sum of M3, M5-1, and M5-3) contributed approximately 34.5% of the total body clearance, second only to M4 (62.6%) (Supplementary Table 1). Therefore, identifying the enzymes responsible for their production is important in guiding future clinical drug interaction studies.

In vitro CYP reaction phenotyping experiments (Fig. 5, Table 1) showed that CYP2B6 primarily catalyzed the formation of M7-1 and M7-2. Both CYP1A2 and CYP2B6 contributed to the formation of M7-3. Although ketoconazole (CYP3A inhibitor) inhibited the production of M7-1 by approximately 24.7%, this may be due to its off-target inhibitory effect on CYP2B6 (Supplementary Table 4). Several studies have also reported that ketoconazole could inhibit other CYP enzymes at a single concentration (Khojasteh et al., 2011; Doran et al., 2022).

UGT1A9 predominantly catalyzed the formation of M4 and M5-3 according to both the recombinant UGT screening data and the inhibition studies with niflumic acid (CYP1A9 inhibitor) (Fig. 6A, 6C and 6E). UGT1A9 and several UGT2B enzymes exhibited catalytic activity of forming M5-1 (Fig. 6B). UGT1A9, UGT2B4, and UGT2B7 were able to catalyze the formation of M5-2 (Fig. 6D). UGT2B isoforms were considered to primarily catalyze the generation of M5-1 and M5-2 (Fig. 6F) although the contribution of individual UGT2B isoforms could not be assessed.
due to the lack of selective inhibitors.

Although the gastrointestinal UGT enzyme UGT1A7 was also able to catalyze the formation of M4, its relative catalytic activity was less than 5% of UGT1A9 (Fig. 6A). Moreover, their protein concentrations in the gastrointestinal tract were low (Harbourt et al., 2012; Sato et al., 2014). Thus, we did not probe into the gastrointestinal glucuronidation in HSK3486 clearance. However, the protein levels of UGT1A9 were high in the kidney, approximately three-fold that in the liver (Harbourt et al., 2012; Sato et al., 2014). Renal glucuronidation is considered to represent the major contributor to HSK3486 extrahepatic metabolism. Knowledge relating to the hepatic and renal contribution to HSK3486 metabolism can help to guide dosage adjustments in patients with liver or renal injury and patients undergoing organ transplantation.

In both incubation systems with or without 2% BSA, the CL_{int,u} values for M4 formation in HKMs were approximately three-fold higher than those in HLMs (Table 3); this was consistent with the relative UGT1A9 amount in human kidneys and liver. However, the hepatic UGT CL_{int,u} value (52.8 mL/min/kg) was significantly underestimated compared to the total hepatic CYP CL_{int,u} value (921 mL/min/kg) when BSA was omitted. Microsomal fatty acids were thought to exhibit competitive inhibition towards UGT1A9 and UGT2B7 (Tsoutsikos et al., 2004; Rowland et al., 2007; Rowland et al., 2008a). Some studies recommended the incorporation of fatty acid-free BSA to sequestrate fatty acids and increase the activity of UGT1A9 and UGT2B7 (Rowland et al., 2008a; Rowland et al., 2008b; Rowland et al., 2009; Walsky et al., 2012; Zientek and Youdim, 2015; Badee et al., 2019).

Upon incorporation of 2% BSA, hepatic and renal UGT CL_{int,u} values were increased by 19 and 24-fold, respectively (Table 3), while the total hepatic CYP CL_{int,u}
values were increased by three-fold (Table 2). The scaled UGT CL_{int,sc,u} values were 845 and 204 mL/min/kg for the liver and kidney, respectively. The scaled total hepatic CYP CL_{int,sc,u} value was 2376 mL/min/kg. Thus, the fractions of the hepatic UGT-mediated metabolic pathway (M4) and the hepatic CYP-mediated metabolic pathway for HSK3486 (M7-1–M7-3) were calculated to be 26.2% and 73.8%, respectively. Applying the well-stirred model, the extrapolated hepatic and renal clearance were 19.3 and 8.81 mL/min/kg, respectively. Renal metabolic clearance accounted for approximately 31.4% of the combined clearance, thus indicating the critical role of renal-mediated glucuronidation in the clearance of HSK3486 in vivo (Table 4).

In conclusion, the structures of major conjugated metabolites (M5-1–M5-3, M3) along with the mono-hydroxylated metabolites (M7-1–M7-3) detected were fully characterized, and more definitive metabolic pathways of HSK3486 in human were determined in this study (Fig. 4). Based on results from the human radiolabeled mass balance study (Bian et al., 2021) and the current study, it was first to elucidate that the M7-1 formation (the sum of downstream metabolites) contributed approximately 34.5% of total body clearance and represented another important metabolic pathway, which is second only to the M4 formation (62.6%). In vitro reaction phenotyping experiments showed that UGT1A9 and CYP2B6 were responsible for forming M4 and M7-1, respectively. These findings would draw attention to the impact of CYP2B6- and UGT1A9-mediated drug interactions and gene polymorphisms with regard to the clinical pharmacokinetics of HSK3486. In addition, IVIVE analysis indicated that renal-mediated glucuronidation was also an essential step in the elimination of HSK3486 from the human body.
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Data Availability Statement

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

Participated in research design: Zhou, Zhu, Tang, Chen.


Contributed new reagents or analytic tools: Zhou, Dong, Fan, Tang, Chen.

Performed data analysis: Zhou, Zhu, Chen.

Wrote or contributed to the writing of the manuscript: Zhou, Zhu, Chen.

Conflicts of interest

The authors declare no conflicts of interest.
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Footnotes

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Figure legends

Fig. 1. Structures of Propofol (A), Ciprofol (HSK3486) (B), and HSK3486-1-O-β-glucuronide (C).

Fig. 2. (A) Metabolic profiles of HSK3486 in HLMs fortified with NADPH; UPLC/Q-TOF MS analysis of CV5-18-05-1 (M7-1) (B), HE6-107-59-1 (M7-2) (C), HE6-44-43-1 (D), HE6-107-52-1 (M7-3) (E), and HE6-44-29-1B (F); MS/MS spectra of M7-1 (G), M7-2 (H), and M7-3 (I) formed in HLMs. MS/MS spectra and fragment patterns of the five mono-hydroxylated HSK3486 reference standards are shown as inserts.

Fig. 3. UPLC/Q-TOF MS analysis of M5-1, M5-2, and M5-3 in human urine and glucuronides formed by mono-hydroxylated HSK3486 compounds in HLMs fortified with UDPGA. Extracted ion chromatograms of M5-1–M5-3 in 0 - 8 h urine (A), glucuronides formed by CV5-18-05-1 (M7-1) (B), glucuronide formed by HE6-107-59-1 (M7-2) (C), glucuronide formed by HE6-107-52-1 (M7-3) (D); MS/MS spectra of M5-1 (E), M5-3 (F) and M5-2(G).

Fig. 4. Proposed metabolic pathways of HSK3486 in humans. Structures marked in red have been confirmed with the reference standards or NMR analysis. Structures marked in blue have been confirmed indirectly by in vitro metabolism studies. The sulfation site of M3 is most likely to be at the 4-position phenolic hydroxyl group of M7-1.

Fig. 5. Formation of the three mono-hydroxylated metabolites M7-1 (A), M7-2 (B), and M7-3 (C) in 10 human recombinant CYP enzymes. Data are presented as means of duplicate samples.
Fig. 6. UGT phenotyping of major glucuronide conjugated metabolites. Formation of M4 (A), M5-1 (B), M5-3 (C), and M5-2 (D) in 12 human recombinant UGT enzymes. Effect of niflumic acid (UGT1A9 inhibitor) on the formation of M4 and M5-3 (E) or M5-1 and M5-2 (F) in HLMs. Data are presented as means of duplicate samples.

Fig. 7. Enzyme kinetics for HSK3486 mono-hydroxylation to form M7-1 (A), M7-2 (B), and M7-3 (C) in HLMs with 2% BSA (red solid circles) or without 2% BSA (open circles). Eadie-Hofstee plots of M7-1 (D), M7-2 (E), and M7-3 (F) are shown in the bottom panel. Data are presented as the means ± S.D. (n = 6).

Fig. 8. Enzyme kinetics for HSK3486 glucuronidation to form M4 in HLMs (A) and HKMs (B) with 2% BSA (blue solid circles) or without 2% BSA (open circles). Eadie-Hofstee plots of M4 in HLMs (C) and HKMs (D) are shown in the bottom panel. Data are presented as the means ± S.D. (n = 3).
Table 1.

Inhibition of mono-hydroxylated metabolites formation in HLMs with selective CYP chemical inhibitors

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>M7-1</th>
<th>M7-2</th>
<th>M7-3</th>
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<tr>
<td></td>
<td>CYP1A2</td>
<td>CYP2B6</td>
<td>CYP2C19</td>
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<td>(−) (+)</td>
<td>(−) (+)</td>
<td>(−) (+)</td>
</tr>
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<td>M7-1</td>
<td>7.5 4.8</td>
<td>90.4 95.2</td>
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<td>94.2 100</td>
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<tr>
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<td>86.6 69.3</td>
<td>3.7 0</td>
</tr>
</tbody>
</table>

Data are displayed as the mean value of duplicate samples.

(+) represents the values with correction for the off-target inhibitory effect of chemical inhibitors; (−) represents the uncorrected values.
Table 2.

Kinetic parameters for HSK3486 mono-hydroxylation to form M7-1, M7-2, and M7-3 in HLMs

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$K_{m,Hi}$ (μM)</th>
<th>$V_{max,Hi}$ (pmol/min/mg)</th>
<th>$K_{m,Lo}$ (μM)</th>
<th>$V_{max,Lo}$ (pmol/min/mg)</th>
<th>$CL_{int}$ a (μL/min/mg)</th>
<th>$CL_{int,a}$ b (mL/min/kg)</th>
<th>$CL_{int,sc}$ b (mL/min/kg)</th>
<th>$f_{CL,HLM,CYP}$</th>
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<tbody>
<tr>
<td>M7-1 (2-enzyme)</td>
<td>1.32 (0.637)</td>
<td>626 (159)</td>
<td>28.2 (6.92)</td>
<td>1490 (141)</td>
<td>526</td>
<td>800</td>
<td>658</td>
<td>0.869</td>
</tr>
<tr>
<td>M7-2 (MM)</td>
<td>4.01 (0.423)</td>
<td>295 (5.68)</td>
<td></td>
<td></td>
<td>73.5</td>
<td>112</td>
<td>91.8</td>
<td>0.121</td>
</tr>
<tr>
<td>M7-3 (2-enzyme)</td>
<td>5.55 (1.30)</td>
<td>33.4 (6.37)</td>
<td>59.2 (54.8)</td>
<td>12.2 (5.68)</td>
<td>6.22</td>
<td>9.44</td>
<td>7.76</td>
<td>0.010</td>
</tr>
<tr>
<td>Sum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLM with 2% BSA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M7-1 (2-enzyme)</td>
<td>4.74 (1.12)</td>
<td>1090 (153)</td>
<td>107 (36.4)</td>
<td>1309 (111)</td>
<td>242</td>
<td>2211</td>
<td>1818</td>
<td>0.765</td>
</tr>
<tr>
<td>M7-2 (MM)</td>
<td>6.55 (0.286)</td>
<td>431 (3.69)</td>
<td></td>
<td></td>
<td>65.8</td>
<td>600</td>
<td>494</td>
<td>0.208</td>
</tr>
<tr>
<td>M7-3 (2-enzyme)</td>
<td>4.08 (1.35)</td>
<td>33.1 (7.13)</td>
<td>67.1 (32.7)</td>
<td>32.0 (5.96)</td>
<td>8.59</td>
<td>78.4</td>
<td>64.5</td>
<td>0.027</td>
</tr>
<tr>
<td>Sum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data for $K_m$ and $V_{max}$ are presented as the mean (S.E) (n = 6).

2-enzyme: two-enzyme Michaelis-Menten model; MM: one-enzyme Michaelis-Menten model; $V_{max,Hi}$ and $K_{m,Hi}$ represent the “high-affinity/low-capacity” enzyme, $V_{max,Lo}$ and $K_{m,Lo}$ represent the “low-affinity/high-capacity” enzyme.

For 2-enzyme model, intrinsic clearance ($CL_{int}$) is equal to the sum of the “high-affinity/low-capacity” enzyme and the “low-affinity/high-capacity” enzyme.

The unbound fraction of HSK3486 ($f_{u,mic}$) in 0.1 mg/mL HLMs and HLMs contained 2% BSA were 0.66 and 0.11, respectively.
Table 3.

Kinetic parameters for HSK3486 glucuronidation to form M4 in HLMs and HKMs

<table>
<thead>
<tr>
<th></th>
<th>$S_{50}$ ($K_m$)</th>
<th>$S_{50u}$</th>
<th>$V_{max}$</th>
<th>Hill Slope</th>
<th>$CL_{int}$</th>
<th>$CL_{int,u}$</th>
<th>$CL_{int,sc,u}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>μM</td>
<td>pmol/min/mg</td>
<td></td>
<td>μL/min/mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLM (AS)</td>
<td>22.7 (1.41)</td>
<td>14.9</td>
<td>1369 (18.1)</td>
<td>1.32 (0.0725)</td>
<td>34.8</td>
<td>52.8</td>
<td>43.4</td>
</tr>
<tr>
<td>HKM (AS)</td>
<td>22.4 (1.50)</td>
<td>17.9</td>
<td>4470 (62.9)</td>
<td>1.36 (0.0818)</td>
<td>112</td>
<td>141</td>
<td>8.41</td>
</tr>
<tr>
<td>HLM with 2% BSA (MM)</td>
<td>17.9 (1.22)</td>
<td>1.96</td>
<td>2012 (22.3)</td>
<td>1.13</td>
<td>1028</td>
<td>845</td>
<td></td>
</tr>
<tr>
<td>HKM with 2% BSA (MM)</td>
<td>17.3 (1.38)</td>
<td>1.89</td>
<td>6442 (82.9)</td>
<td>373</td>
<td>3407</td>
<td>204</td>
<td></td>
</tr>
</tbody>
</table>

Data for $S_{50}(K_m)$ and $V_{max}$ represent the mean (S.E) (n = 3).

AS: Allosteric sigmoidal model; MM: one-enzyme Michaelis-Menten model.

$^a$ The unbound fraction of HSK3486 ($f_{u,mic}$) in 0.05 mg/mL HKMs, 0.1 mg/mL HLMs, and HKMs or HLMs contained 2% BSA were 0.80, 0.66, and 0.11, respectively.
<table>
<thead>
<tr>
<th>Organ</th>
<th>CYP CL_{int,su}^{a}</th>
<th>UGT CL_{int,su}^{b}</th>
<th>Total CL_{int,su}</th>
<th>CL_{b}^{c}</th>
<th>f_{CL}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>2376</td>
<td>845</td>
<td>3221</td>
<td>19.3</td>
<td>0.686</td>
</tr>
<tr>
<td>Kidney</td>
<td>-</td>
<td>204</td>
<td>204</td>
<td>8.81</td>
<td>0.314</td>
</tr>
<tr>
<td>Combined</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>28.1</td>
<td>1.000</td>
</tr>
</tbody>
</table>

\(^a\) CYP CL_{int,su} value was derived from the total \textit{in vitro} clearance of the three hydroxylated metabolites in HLMs with 2\% BSA.

\(^b\) UGT CL_{int,su} value was derived from the \textit{in vitro} clearance determined in HLMs and HKMs with 2\% BSA.

\(^c\) Organ blood clearance was calculated utilizing eq.8 as described in Data Analysis.
Fig. 1

A  Propofol

B  Ciprofol (HSK3486)

C  M4 (HSK3486-1-O-β-glucuronide)
Fig. 2

A

HSK3496_HLM_NADPH

B

CVS-169-51_MT-1

C

HE5-107-94_MT-2

D

HE5-44-43_MT-3

E

HE5-107-52_MT-3

F

HE5-44-29-1B

G

MS/MS Spectrum of M7-1

H

MS/MS Spectrum of M7-2

I

MS/MS Spectrum of M7-3
Fig. 3

(A) M5-2 Glucuronide

(B) CV5-18-05-1_HLM_UOMPA

(C) HE6-107-59-1_HLM_UOMPA

(D) HE6-107-52-1_HLM_UOMPA

(E) MS/MS Spectrum of M5-1

(F) MS/MS Spectrum of M5-3

(G) MS/MS Spectrum of M5-2
Fig. 4

CYP2B6

HSK3486 → M4

UGT1A9

M7-2

M5-2

CYP2B6

M7-1

M5-3

CYP2B6/CYP1A2

M7-3

M5-1

M2-1, M2-2, M2-3

M10-2

M11-2

M3

M10-1

M11-1

M8-1

M6-1

M9

M8-2

M6-2

GlcA: Glucuronic acid
Fig. 7

A  M7-1

B  M7-2

C  M7-3

D  M7-1

E  M7-2

F  M7-3
Fig. 8

A. Human Liver Microsome

B. Human Kidney Microsome

C.

D.
SUPPLEMENTARY

Cytochrome P450 2B6 and UGT Enzymes-Mediated Clearance of Ciprofol (HSK3486) in Humans: The Role of Hepatic and Extrahepatic Metabolism

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\textsuperscript{d} MassDefect Technologies, Princeton, NJ 08540, USA (M.Z.)
Supplementary Method 1. Analysis of Human Plasma and In Vitro Incubation Samples by UPLC-UV/Q-TOF MS.

HSK3486 metabolites were separated on an Acquity UPLC system (Waters, USA) fitted with an ACQUITY™ HSS T3 C18 column (100 × 2.1 mm, 1.8 μm). The mobile phase consisted of 5 mM ammonium acetate containing 0.001% ammonia (A) and acetonitrile (B). The total gradient elution duration was 15 min: 0 min, 10% B; 1 min, 10% B; 9 min, 80% B; 11 min, 95% B; 12.5 min, 95% B; 13.5 min, 10% B; 15 min, 10% B. To separate the two epimers of M5-2 (PH-HSC-008-0A-1 glucuronide and PH-HSC-008-0C-1 glucuronide), the gradient program was adjusted as follows: 0 min, 0% B; 3 min, 0% B; 10 min, 15% B; 30 min, 15% B; 32 min, 25% B; 45 min, 25% B; 55 min, 80% B; 65 min, 100% B; 70 min, 100% B; 72 min. The column temperature and flow rate were 40°C and 0.4 mL/min, respectively. The wavelength of the TUV detector was set to 280 nm.

High resolution mass spectrum data were acquired by a Synapt G2-Si Q-TOF System (Waters, Manchester, UK) equipped with an electrospray ionization source in negative mode. The capillary voltage and source temperature were set at 2.5 kV and 120°C, respectively. The desolvation gas flow and temperature were set at 800 L/h and 350°C, respectively. The Trap CE and Transfer CE were both set at 1 eV during the low-energy scan to obtain full MS spectrum data. During the high-energy scan, the trap CE was ramped from 5 to 10 eV and the transfer CE was ramped from 10 to 30 eV to obtain fragment ion spectrum data. Further MS/MS experiments were performed for the screened metabolites to obtain their product ion spectra. The scan range of TOF MS was set to \( m/z \ 50 - m/z \ 1000 \) with a scan time of 0.2 s. MS spectrum data were corrected during acquisition using an external reference (Lock-Spray™) consisting of a 50 ng/mL solution of Leu-enkephalin that was infused at 10 μL/min, generating a reference ion at
m/z 554.2615. Masslynx version 4.1 (Waters, USA) was used for UPLC-UV/Q-TOF MS data acquisition, and UNIFI version 1.9.2 (Waters, USA) was used for metabolite screening and identification.

**Supplementary Method 2. Determination of HSK3486 and its Metabolites by LC-MS/MS**

The LC-MS/MS system featured a Nexera X2 UHPLC system (Shimadzu, Kyoto, Japan) and a Triple Quad 5500 mass spectrometer (AB Sciex, MA, USA). M7-1, M7-2, M7-3, M4 and HSK3486 were separated on an ACQUITY BEH C18 column (50 × 2.1 mm, 1.7 μm). The mobile phases included 10 mM of ammonium acetate containing 0.001% ammonia (phase A) and acetonitrile (phase B). The gradient elution program was as follows: 0 min, 10% B; 0.2 min, 10% B; 1.5 min, 98% B; 2.5 min, 98% B; 3.0 min, 10% B; 3.2 min, 10% B. The column temperature and flow rate were set to 40 °C and 0.6 mL/min, respectively.

Data acquisition was performed in negative mode. The ion spray voltage and the source temperature were set to -4500 V and 500°C, respectively. The pressures of gas 1, gas 2, and the curtain gas were set to 50 psi, 50 psi, and 35 psi, respectively. The following MRM transitions were monitored: M7-1 (m/z 219.1 → m/z 177.1), M7-2 (m/z 219.1 → m/z 189.0), M7-3 (m/z 219.1 → m/z 161.1), M4 (m/z 379.1 → m/z 203.1), M5-1 (m/z 395.2 → m/z 219.0), M5-2 (m/z 395.2 → m/z 193.1), M5-3 (m/z 395.2 → m/z 219.0), HSK3486 (m/z 203.1 → m/z 175.1), mefenamic acid (m/z 239.6 → m/z 196.0), and propofol (m/z 177.0 → m/z 161.1). The calibration standard ranges were 10 - 1000 nM for M7-1, 1.5 - 150 nM for M7-2, 0.5 - 100 nM for M7-3, 5 - 5000 nM for M4, and 0.1 - 100 μM for HSK3486, respectively. The area ratio (analyte peak area/ internal standard peak area) was also monitored for M5-1, M5-2, and M5-3.

To quantify M7-1, M7-2, and M7-3, 65 μL of each sample was mixed with 65 μL
of acetonitrile/water (7:3, v/v) solution containing 500 ng/mL of mefenamic acid. After vortexing at 1000 rpm for 15 min and centrifuging at 2250 g for 15 min, 10 µL of supernatant was taken for LC-MS/MS analysis. To quantify M4, 25 µL of each sample was mixed with 100 µL of acetonitrile containing 500 ng/mL of mefenamic acid. After vortexing and centrifuging, 3 µL of supernatant was taken for LC-MS/MS analysis. To quantify HSK3486, 25 µL of each sample was mixed with 100 µL of acetonitrile containing 1 µg/mL of propofol. After vortexing and centrifuging, 15 µL of supernatant was taken for LC-MS/MS analysis. To analyze M5-1, M5-2, and M5-3 in UGT phenotyping studies, 50 µL of each sample was mixed with 150 µL of acetonitrile and 50 µL of 250 ng/mL of mefenamic acid. After vortexing at 1000 rpm for 15 min and centrifuging at 2250 g for 15 min, 225 µL of supernatants were dried with nitrogen flow at 40 °C. The residue was dissolved in 120 µL of acetonitrile-water (1:3) and a 15-µL aliquot of the supernatant was taken for LC-MS/MS analysis. LC-MS/MS data were collected and analyzed using Analyst version 1.6.3 software.
Supplementary Table 1. Normalization of excretion data of HSK3486 and its metabolites after a single intravenous administration of 0.8 μCi/0.4 mg/kg [14C] HSK3486 injectable emulsion to healthy subjects (n=6)

<table>
<thead>
<tr>
<th>Metabolic Pathway</th>
<th>Formula change</th>
<th>ID</th>
<th>Detected (% Dose)a</th>
<th>Percent of total radioactivity (%)</th>
<th>fCL</th>
<th>Sum %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucuronidation</td>
<td>+C₆H₈O₆</td>
<td>M4</td>
<td>51.6</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>+O</td>
<td>M7</td>
<td>N.D.</td>
<td>0.2</td>
<td>N.D.</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>+O+SO₃</td>
<td>M3</td>
<td>2.3</td>
<td>0.1</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>+O+C₆H₈O₆</td>
<td>M5-1</td>
<td>19.3</td>
<td>N.D.</td>
<td>23.4</td>
<td>23.4</td>
</tr>
<tr>
<td></td>
<td>+O+C₆H₈O₆</td>
<td>M5-2</td>
<td>0.3</td>
<td>N.D.</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>+O+C₆H₈O₆</td>
<td>M5-3</td>
<td>6.9</td>
<td>N.D.</td>
<td>8.3</td>
<td>8.3</td>
</tr>
<tr>
<td>Oxidationb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mono-hydroxylation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+O</td>
<td>M7</td>
<td>N.D.</td>
<td>0.2</td>
<td>N.D.</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>+O+SO₃</td>
<td>M3</td>
<td>2.3</td>
<td>0.1</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>+O+C₆H₈O₆</td>
<td>M5-1</td>
<td>19.3</td>
<td>N.D.</td>
<td>23.4</td>
<td>23.4</td>
</tr>
<tr>
<td></td>
<td>+O+C₆H₈O₆</td>
<td>M5-2</td>
<td>0.3</td>
<td>N.D.</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>+O+C₆H₈O₆</td>
<td>M5-3</td>
<td>6.9</td>
<td>N.D.</td>
<td>8.3</td>
<td>8.3</td>
</tr>
<tr>
<td>Multi-step oxidation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.8</td>
<td>N.D.</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Total radioactive peaks identified</td>
<td></td>
<td></td>
<td>82.2</td>
<td>0.3</td>
<td>82.5</td>
<td>2.2</td>
</tr>
</tbody>
</table>

a Dose excreted data of HSK3486 metabolites were obtained from (Br J Clin Pharmacol, 2021, 87:93-105).

b Overall oxidation pathways were divided into two groups: including mono-hydroxylation and multi-step oxidation. The mono-hydroxylation pathway was calculated as the recovered mono-hydroxylated metabolite M7 (0.2% of dose) and sequential conjugated metabolites, including sulfate conjugated metabolite M3 (2.3% of dose) and glucuronide conjugated metabolites M5-1 (19.3% of dose), M5-2 (0.3% of dose), M5-3 (6.9% of dose). The Multi-step oxidation was calculated as nine glucuronide conjugates of the multi-step oxidative metabolites recovered in urine, accounting for 1.8% of the dose administrated.
**Supplementary Table 2. Positive Control Data of Corning Supersomes Cytochrome P450 enzymes**

<table>
<thead>
<tr>
<th>Supersomes</th>
<th>Substrate</th>
<th>Marker reaction</th>
<th>Incubation Concentration</th>
<th>Enzyme Activity pmol/min/pmol P450</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin</td>
<td>Phenacetin O-deethylation</td>
<td>50</td>
<td>21.2</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Coumarin</td>
<td>Coumarin 7-hydroxylation</td>
<td>400(\textsuperscript{a})</td>
<td>50.0</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Bupropion</td>
<td>Bupropion hydroxylation</td>
<td>100</td>
<td>4.86</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Amodiaquine</td>
<td>Amodiaquine N-deethylation</td>
<td>5</td>
<td>48.3</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac</td>
<td>Diclofenac 4’-hydroxylation</td>
<td>200</td>
<td>56.0</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-Mephenytoin</td>
<td>S-Mephenytoin 4’-hydroxylation</td>
<td>100</td>
<td>27.0</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan</td>
<td>Dextromethorphan O-demethylation</td>
<td>10</td>
<td>82.2</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>p-Nitrophenol</td>
<td>p-Nitrophenol hydroxylation</td>
<td>500(\textsuperscript{a})</td>
<td>16.0</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Testosterone</td>
<td>Testosterone 6β-hydroxylation</td>
<td>100</td>
<td>238</td>
</tr>
<tr>
<td>UGT3A5</td>
<td>Testosterone</td>
<td>Testosterone 6β-hydroxylation</td>
<td>100</td>
<td>11.2</td>
</tr>
</tbody>
</table>

Data are presented as the mean value of duplicate samples.

\(\textsuperscript{a}\) The Coumarin 7-hydroxylation activity (CYP2A6) and p-Nitrophenol hydroxylation (CYP2E1) were provided by the vendors.
**Supplementary Table 3. Positive Control Data of Corning Supersomes Human UDP-glucuronosyltransferases**

<table>
<thead>
<tr>
<th>Supersomes</th>
<th>Substrate</th>
<th>Glucuronidation site</th>
<th>Incubation Concentration μM</th>
<th>Enzyme Activity pmol/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A1</td>
<td>β-estradiol</td>
<td>3β-OH</td>
<td>50</td>
<td>700</td>
</tr>
<tr>
<td>UGT1A3</td>
<td>β-estradiol</td>
<td>3β-OH</td>
<td>100</td>
<td>54.1</td>
</tr>
<tr>
<td>UGT1A4</td>
<td>Trifluoperazine</td>
<td>Piperazine N4</td>
<td>200</td>
<td>1057</td>
</tr>
<tr>
<td>UGT1A6</td>
<td>4-Methylumbelliferone</td>
<td>7-OH</td>
<td>100</td>
<td>6510</td>
</tr>
<tr>
<td>UGT1A7</td>
<td>4-Methylumbelliferone</td>
<td>7-OH</td>
<td>15</td>
<td>2380</td>
</tr>
<tr>
<td>UGT1A8</td>
<td>β-estradiol</td>
<td>3β-OH</td>
<td>50</td>
<td>233</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>Mycophenolic acid</td>
<td>4-OH</td>
<td>100</td>
<td>14100</td>
</tr>
<tr>
<td>UGT1A10</td>
<td>β-estradiol</td>
<td>3β-OH</td>
<td>5</td>
<td>262</td>
</tr>
<tr>
<td>UGT2B4</td>
<td>Gemfibrozil</td>
<td>1-COOH</td>
<td>100</td>
<td>181</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>Gemfibrozil</td>
<td>1-COOH</td>
<td>5</td>
<td>202</td>
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<tr>
<td>UGT2B15</td>
<td>4-Methylumbelliferone</td>
<td>7-OH</td>
<td>100</td>
<td>632</td>
</tr>
<tr>
<td>UGT2B17</td>
<td>β-estradiol</td>
<td>17β-OH</td>
<td>10</td>
<td>30.0</td>
</tr>
</tbody>
</table>

Data are presented as the mean value of duplicate samples.
**Supplementary Table 4. Off-target inhibition effect of chemical inhibitors on CYP marker reactions**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Selectivity of inhibitors on CYP marker reactions (percent inhibition)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phenacetin CYP1A2</td>
<td>Bupropion CYP2B6</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>α-Naphthoflavone</td>
<td>84.2%</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Ticlopidine</td>
<td>9.9%</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>N-benzylpirvanol</td>
<td>0.0%</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Ketoconazole</td>
<td>8.8%</td>
</tr>
</tbody>
</table>

Data are presented as the mean value of duplicate samples.
Representative metabolic profiles of HSK3486 in human plasma and urine. (A) 4 - 15 min pooled plasma without concentration treatment; (B) 4 - 15 min pooled plasma with concentration treatment; (C) 0 - 8 h pooled urine with concentration treatment; (D) 24 - 48 h pooled urine with concentration treatment.
Supplementary Fig. 2.

UPLC/Q-TOF MS analysis of M3 detected in human urine and sulfates formed by mono-hydroxylated HSK3486 compounds in HLS9s fortified with PAPS. Extracted ion chromatograms of M3 in 0-8 h urine (A), sulfate formed by CV5-18-05-1 (M7-1) (B), sulfate formed by HE6-107-59-1 (M7-2) (C), no sulfate was formed by HE6-107-52-1 (M7-3).
Supplementary Fig. 3.

\(^{13}\)C-\(^{1}\)H HMBC spectrum of M5-1 (A) and M5-2 (B). The arrows in the figure represent HMBC corrections.
Supplementary Fig. 4.

Separation of two epimers of M5-2 in human urine. (A) Extracted ion chromatogram of M5-2 in 0 - 8 h urine analyzed with a modified UPLC method; (B) Overlay extracted ion chromatogram of glucuronides formed by PH-HSC-008-0A-1 and PH-HSC-008-0C-1 (two enantiomers of M7-2 [HE6-107-59-1]) in HLMs fortified with UDPGA.
Supplementary Fig. 5.

Metabolic profiles of M7-1 (A), M7-2 (B), and M7-3 (C) in HLS9s fortified with NADPH and UDPGA.
NMR spectra of Metabolite M5-1 and M5-2

A. Metabolite M5-1

Molecular Formula: C20H28O8  
Average Mass: 396.4360  
Monoisotopic Mass: 396.1784

HRMS (UPLC/QTOF MS) \textit{m}/\textit{z}: [M-H] Calcd for C20H27O8 395.1711; Found 395.1716.

$^1$H NMR (500 MHz, Methanol-$d_4$) $\delta$ 6.98 (d, $J = 2.8$ Hz, 1H), 6.86 (d, $J = 2.8$ Hz, 1H), 4.79 (d, $J = 6.7$ Hz, 1H), 3.87 (s, 1H), 3.63 (s, 1H), 3.51 (q, $J = 9.1$, 6.8 Hz, 2H), 3.30 (q, $J = 6.9$ Hz, 1H), 2.51 (dq, $J = 9.1$, 6.9 Hz, 1H), 1.27 (d, $J = 6.9$ Hz, 3H), 1.22 (d, $J = 6.8$ Hz, 6H), 1.04 (pd, $J = 8.5$, 7.5, 3.3 Hz, 1H), 0.57 (tt, $J = 8.9$, 4.6 Hz, 1H), 0.39 (tt, $J = 8.9$, 4.7 Hz, 1H), 0.21 (dq, $J = 9.4$, 4.8 Hz, 1H), 0.13 (dq, $J = 9.4$, 4.9 Hz, 1H).
$^{13}$C NMR (126 MHz, MeOD) δ 153.19, 147.31, 138.30, 137.26, 115.06, 113.78, 104.15, 77.65, 74.79, 73.35, 38.75, 28.09, 23.71, 23.41, 21.27, 18.36, 5.26, 4.22.
B. Metabolite M5-2 (PH-HSC-008-0A-1 glucuronide)

Molecular Formula: C20H28O8
Average Mass: 396.4360
Monoisotopic Mass: 396.1784

HRMS (UPLC/QTOF MS) \( m/z \): [M-H] Calcd for C20H27O8 395.1711; Found 395.1713.

\(^1\)H NMR (500 MHz, Methanol-\(d_4\)) \( \delta \) 7.17 (dd, \( J = 7.5, \) 1.6 Hz, 1H), 7.04 – 7.00 (m, 1H), 6.86 (t, \( J = 7.6 \) Hz, 1H), 4.39 (d, \( J = 7.6 \) Hz, 1H), 3.92 (t, \( J = 8.4 \) Hz, 1H), 3.77 – 3.70 (m, 1H), 3.55 (s, 1H), 3.53 – 3.48 (m, 1H), 3.41 (s, 1H), 3.26 (dd, \( J = 15.1, \) 6.8 Hz, 1H), 2.51 (m, 1H), 1.31 (d, \( J = 3.5 \) Hz, 3H), 1.29 (d, \( J = 2.6 \) Hz, 3H), 1.28 (d, \( J = 2.6 \) Hz, 3H), 1.06 (m, 1H), 0.61 – 0.52 (m, 1H), 0.38 (m, 1H), 0.21 (m, 1H), 0.13 (m, 1H).
$^{13}$C NMR (126 MHz, MeOD) $\delta$ 152.57, 136.18, 132.23, 126.23, 125.31, 121.53, 104.63, 77.57, 76.62, 74.76, 73.26, 38.71, 34.29, 21.38, 18.39, 17.76, 5.33, 4.32.

$^{13}$C-$^1$H HSQC

Methanol-$d_4$