

Research Article

LC–MS/MS-Based Quantitative Investigation of the Exposure Characteristics of Psilocin, the Active Metabolite of Psilocybin, in Rats

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Abstract

Background: Psilocybin has shown promising antidepressant effects; however, pharmacokinetic data on psilocybin and the associated active metabolite, psilocin, remain limited. Thus, this study aimed to develop and validate a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for quantifying psilocin and to investigate the associated pharmacokinetics, tissue distribution, excretory characteristics in rats, and transmembrane transport properties. **Methods:** An LC–MS/MS method for determining psilocin in rat plasma was developed and validated. Male Sprague-Dawley (SD) rats received intravenous (i.v.) psilocybin (1 mg/kg) or oral psilocybin at low, medium, and high doses (1, 3, and 10 mg/kg, respectively). Plasma psilocin concentrations were measured serially. The medium-dose group (3 mg/kg psilocybin) included both male and female rats to evaluate sex-related differences. In male rats administered 3 mg/kg psilocybin orally, tissue samples were collected at various time points to quantify psilocin concentrations across numerous tissues. Following oral administration of 10 mg/kg psilocybin to male rats, excretory samples were collected over time to characterize the excretion profile of psilocin. Additionally, transmembrane transport was assessed using Caco-2 cells and LLC-PK1 cells overexpressing human MDR1 (hMDR1). **Results:** The bioanalytical method employed met all validation criteria. Following intravenous administration, psilocin demonstrated a clearance of 4.4 ± 0.4 L/h/kg and an volume of distribution at steady state of 1.5 ± 0.1 L/kg. Oral administration of psilocybin resulted in nonlinear pharmacokinetics; dose-normalized psilocin area under the concentration–time curve (AUC) increased by 63% (medium dose) and 102% (high dose) compared to the low-dose group. No statistically significant differences in pharmacokinetic parameters were observed between male and female subjects. After oral administration of the medium dose, psilocin was extensively distributed to the brain and major tissues, with tissue-to-plasma partition coefficient (K_p) values exceeding 1 in all cases (excluding fat). Psilocin exhibited moderate permeability *in vitro* ($P_{app} = 12.39 \pm 1.31 \times 10^{-6}$ cm/s). In P-glycoprotein (P-gp)-expressing monolayer cell models, the efflux ratio was 1.26, which is below the threshold of 2, indicating that psilocin is not a P-gp substrate. **Conclusions:** Psilocybin is rapidly metabolized to psilocin *in vivo*. The pharmacokinetic profile of psilocin is characterized by rapid clearance, extensive tissue distribution, nonlinear kinetics, and low cumulative recovery. The favorable membrane permeability and P-gp-mediated efflux resistance of psilocin likely contribute to efficient penetration of the blood–brain barrier and broad tissue distribution.

Keywords: psilocybin; psilocin; LC-MS/MS; pharmacokinetics; tissue distribution; excretion

1. Introduction

Depression is a highly prevalent mental disorder, affecting over 350 million people globally throughout their lifetime. It not only contributes to cognitive impairment and increased suicide risk among patients, but also ranks among the top three noncommunicable diseases in terms of overall disease burden. This burden is driven by reduced work-force participation and elevated healthcare costs, making depression a condition that harms both individuals and society [1]. The pathophysiology of depression has long been associated with dysregulation of monoamine neurotransmitters, such as serotonin, norepinephrine, and dopamine [2]. Current clinical treatment relies primarily on selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs). While these

agents can alleviate symptoms in some patients, they present several limitations: slow onset of action (often requiring several weeks), low response rates, and side effects such as sexual dysfunction and sedation. These drawbacks limit their ability to meet diverse clinical needs [3].

In recent years, classical psychedelic drugs such as psilocybin have shown significant therapeutic potential for mental health disorders [4], with research progressing rapidly. Several Phase II clinical trials have demonstrated that psilocybin produces rapid and sustained antidepressant effects in patients with treatment-resistant depression [5,6,7]. In light of these promising results, the U.S. FDA has granted psilocybin Breakthrough Therapy designation for both treatment-resistant depression and major depressive disorder, recognizing its substantial clinical value [8].



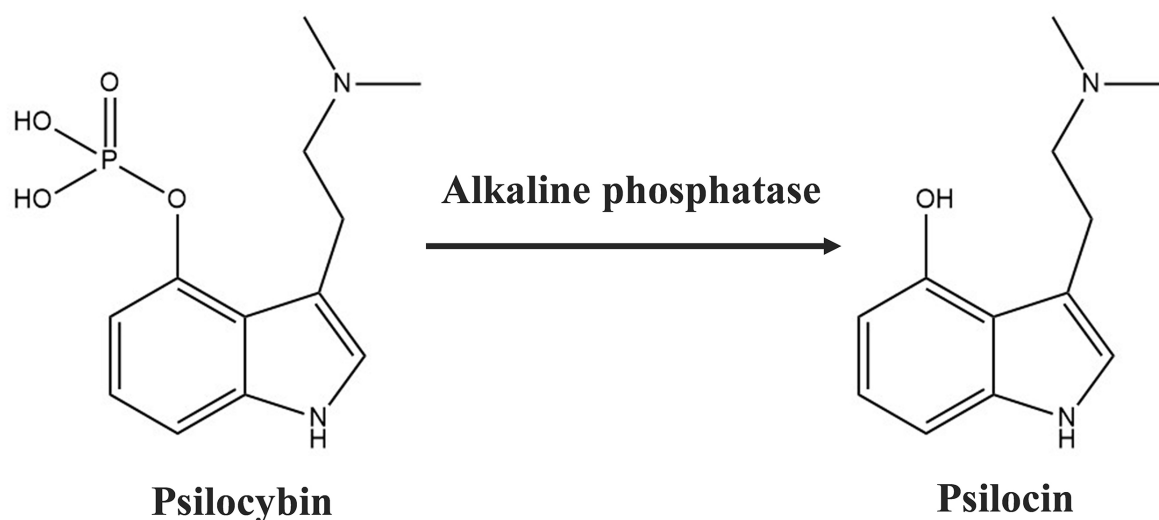


Fig. 1. The structure and metabolic pathway of psilocybin to psilocin.

Psilocybin (4-phosphoxy-N, N-dimethyltryptamine) is a naturally occurring serotonergic hallucinogen produced by mushrooms of the genus *Psilocybe* [9,10]. As illustrated in Fig. 1, following *in vivo* administration, psilocybin is rapidly dephosphorylated by alkaline phosphatase or nonspecific esterases to form its active metabolite, psilocin (4-hydroxy-dimethyltryptamine). Psilocin is subsequently metabolized primarily via monoamine oxidase-A (MAO-A), cytochrome P450 enzymes (CYP2D6/3A4), and UDP-glucuronosyltransferases (UGT1A9/1A10) [11]. Structurally, psilocin closely resembles serotonin. Unlike traditional antidepressants such as SSRIs, which indirectly modulate neurotransmission by increasing synaptic serotonin levels, psilocin acts as a direct agonist at the 5-HT_{2A} receptor. This activation promotes neuroplastic adaptations and enhances cortical neurogenesis, leading to rapid antidepressant effects [12,13,14,15].

The pharmacokinetics of psilocybin and its active metabolite psilocin remain inadequately characterized. Critical parameters, such as dose-dependent pharmacokinetic profiles, tissue distribution patterns, excretion characteristics, transmembrane transport mechanisms, and potential sex differences have yet to be fully elucidated. This knowledge gap limits a comprehensive understanding of psilocybin's antidepressant effects, thereby hindering the optimization of clinical dosing regimens and the development of personalized therapeutic strategies.

To address these knowledge gaps, we developed and validated a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for quantifying psilocin in biological matrices. This method was applied to characterize the pharmacokinetic profile, tissue distribution, excretion patterns, and transmembrane transport mechanisms of psilocin-the active metabolite of psilocybin.

2. Materials and Methods

2.1 Materials

Psilocybin (CAS, 520-52-5, purity $\geq 96\%$) and psilocin hydrochloride (CAS, 520-53-6, purity $\geq 98.6\%$) were synthesized by the Academy of Military Medical Sciences (Beijing, China). Glipizide (Cat, G70920, purity $\geq 98\%$, internal standard) was purchased from Acme Biochemical Technology Co., Ltd. (Shanghai, China). Dimethyl sulfoxide (Cat, M75382, purity $\geq 99.7\%$) was purchased from Meryer Chemical Technology Co., Ltd. (Shanghai, China). Acetonitrile (Cat, A998-4, chromatography grade) was purchased from Thermo Fisher (Shanghai, China).

Digoxin (Cat, S4290), Atenolol (Cat, S4817), metoprolol (Cat, S5430) and Topotecan HCl (Cat, S1231) were purchased from Selleckchem (Houston). RPMI 1640 (Cat, C11875500CP), fetal bovine serum (Cat, A5256701), and penicillin-streptomycin solution (Cat, 15140122) were purchased from Gibco (Shanghai, China). Hanks' balanced salt solution (HBSS) (Cat, H1025) was purchased from Solarbio (Beijing, China); Transwell cell culture plates and inserts (Cat, 3412) were purchased from Corning (Shanghai, China). Cell resistance meter was purchased from Beijing KingTech Technology Co., Ltd. (Beijing, China).

Phoenix WinNonlin (version 8.1) was purchased from Certara (Princeton, NJ, USA). GraphPad Prism (version 10.1) was purchased from GraphPad Software (Boston, MA, USA). ChemDraw (version 23) was purchased from CambridgeSoft (Cambridge, MA, USA).

2.2 Animals and Cells

Sprague-Dawley rats (200–230 g; male and female) were obtained from Beijing Charles River Laboratory Animal Technology Co., Ltd. All animals were housed under standard laboratory conditions in accordance with animal welfare guidelines: ambient temperature was maintained

at 22 ± 2 °C, relative humidity at 45%–60%, and a 12 h light/dark cycle was implemented. Rats had free access to standard rodent chow and water throughout the study.

Animals were of specific pathogen-free (SPF) grade, and their health status was confirmed. They were wild-type, without genetic modification, and no genotyping was performed. All animals were experimentally naïve, with no prior procedures. They were acclimatized for 1 week.

Caco-2 cells were purchased from the American Type Culture Collection (ATCC, USA). LLC-PK1 cells overexpressing the human MDR1 (hMDR1) transporter (LLC-PK1-MDR1) and LLC-PK1 cells transfected with a blank plasmid (LLC-PK1-MOCK) were gifted by Professor Qingcheng Mao at the University of Washington. Caco-2 cells were maintained in 88% DMEM low-glucose (Gibco, 11885084) supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin (PS), and 1% MEM non-essential amino acids (NEAA, 11140-050). LLC-PK1 mock and MDR1 cells were cultured in 89% RPMI 1640 medium containing 10% FBS and 1% PS. Both cell lines were incubated at 37 °C in a humidified atmosphere with 5% CO₂. Cells were used at passages below 20 for Caco-2 and below 10 for LLC-PK1 (mock/MDR1). All cell lines were validated by STR profiling and tested for mycoplasma contamination using the Mycolor One-Step Mycoplasma Detector and were found to be negative.

In the animal experiments, a single rat served as the experimental unit; in the *in vitro* experiments, a single Transwell well served as the experimental unit. A sample size of 6 animals per group or 3 wells (for cell-based assays) is a conventional sample size in pharmacokinetic studies, as determined based on the ICH M10 guideline and common practice in the literature.

Pharmacokinetics: four male groups (intravenous 1 mg/kg; oral 1, 3, and 10 mg/kg) plus one female group (oral 3 mg/kg), $n = 6$ per group, 30 animals in total. Tissue distribution: five time points (15 min, 1, 4, 6, and 12 h), $n = 6$ per time point, 30 male animals in total (separate cohort). Excretion studies: urine/feces in 6 animals and bile in 6 animals, 12 male animals in total (separate cohort). Total animals: 72 (66 males + 6 females).

P-gp efflux assay: 2 cell lines (MOCK/MDR1) $\times$ 2 directions (A $\rightarrow$ B/B $\rightarrow$ A) $\times$ 3 replicates = 12 wells (test article); metoprolol, atenolol, and digoxin were tested in the same manner (12 wells each); 48 wells in total. Caco-2 transport assay: psilocin (2 directions $\times$ 3 replicates = 6 wells) + metoprolol (6 wells) + atenolol (6 wells) + digoxin (6 wells) = 24 wells.

In the pharmacokinetic study (24 male rats) and the tissue distribution study (30 male rats), animals were assigned to groups using a random number table. For female rats, only a single oral mid-dose group was included; therefore, no randomization was involved. The excretion study was conducted as two independent batches; no inter-group randomization was involved, but cage positions were ran-

domly arranged. For cell-based experiments, wells were assigned using a randomized block design.

Animal housing conditions were standardized. Samples were processed uniformly and analyzed in the same batch, with the LC-MS/MS injection order following animal number. Uncontrolled factors: Blinding was not applied; however, because all endpoints were objective concentration values and involved no subjective judgment, the impact is considered minimal.

2.3 LC-MS/MS Detection Conditions and Sample Preparation

Accurate aliquots of 10 mg/mL stock solutions of psilocin and glipizide were separately transferred. Each solution was serially diluted with acetonitrile to achieve a final concentration of 100 ng/mL. These diluted solutions were used to establish the liquid chromatography–tandem mass spectrometry (LC-MS/MS) analytical method.

The concentration of psilocin in various biological samples was determined using high-performance liquid chromatography (HPLC-40AD) coupled with a triple quadrupole mass spectrometer (Shimadzu 8050, Japan). Chromatographic separation was achieved on a Kinetex-C18 column (3 mm $\times$ 50 mm, 2.6 μ m) maintained at 40 °C. The mobile phase comprised (A) an aqueous solution containing 0.1% formic acid and (B) an acetonitrile solution containing 0.1% formic acid, with the following gradient program: 3% B from 0.1 to 0.5 min; increased linearly from 3% to 97% B between 0.5 and 2.3 min; held at 97% B from 2.3 to 3.3 min; decreased to 65.7% B by 3.4 min; further reduced to 3% B by 3.6 min; and re-equilibrated at 3% B from 3.6 to 4.4 min. The injection volume was 5 μ L.

Mass spectrometric detection was performed using an electrospray ionization (ESI) source in positive ion mode. Operation parameters were set as follows: drying gas and heating gas flow rates, 10 L/min each; collision gas pressure, 270 kPa; ion source temperature, 200 °C; desolvation line temperature, 200 °C; and heating module temperature, 400 °C. Quantification was carried out via multiple reaction monitoring (MRM) using the transition m/z 205.30 $\rightarrow$ 58.20 for psilocin and m/z 446.30 $\rightarrow$ 321.15 for glipizide as the internal standard.

All biological samples in this study were pretreated via protein precipitation. The specific procedures were as follows: Plasma samples: A 20 μ L aliquot of rat plasma was mixed with 20 μ L of acetonitrile, followed by 200 μ L of acetonitrile containing 10 ng/mL glipizide (internal standard). The mixture was vortexed for 1 min and centrifuged at 18,000 $\times g$ for 10 min. The resulting supernatant was collected for LC-MS/MS analysis. Tissue samples: Tissue homogenates were prepared using an extraction solvent at a 1:4 (w/v) ratio. Liver and kidney tissues were homogenized in pure acetonitrile, while other tissues were homogenized in 50% acetonitrile. Subsequently, an aliquot equivalent to 20 μ L of plasma volume was taken from each homogenate

and processed identically to the plasma pretreatment protocol described above. Cell experiment samples: Samples from cell-based experiments were processed using the same pretreatment method as applied to plasma samples. Euthanasia was performed by gradually increasing the CO₂ concentration in the chamber to >90% and maintaining it for 5–6 min; the CO₂ used had a purity $\geq 99\%$.

2.4 Methodological Validation of Psilocin Quantification in Rat Plasma

The rat plasma methodology was validated in accordance with ICH harmonized guidance M10 on bioanalytical method validation and sample analysis to ensure reliable testing. All samples for the validation were analyzed following the aforementioned sample preparation procedure.

2.4.1 Selectivity

Double blank (DB) matrix samples, containing neither psilocin nor glipizide, were prepared using blank plasma pooled from six individual rat sources. To establish the lower limit of quantitation (LLOQ) calibration point, a working solution of psilocin was spiked at the LLOQ concentration into six separate aliquots of blank rat plasma from individual sources. All samples, including calibration standards, were then protein-precipitated using a precipitant solution containing the internal standard. Additionally, study samples were obtained from rat plasma collected 15 minutes after the oral administration of 3 mg/kg psilocybin.

2.4.2 Calibration Curve and Limit of Quantification

Based on the plasma concentrations from the rat PK pilot study, the linear range for psilocin in rat plasma was determined to be 5–500 ng/mL, with a lower limit of quantification of 5 ng/mL.

2.4.3 Accuracy and Precision

Quality control (QC) samples were prepared at concentrations corresponding to the lower limit of quantification (LLOQ) and low, medium, and high levels (5, 15, 150, and 400 ng/mL; $n = 6$ per level). In three independent analytical batches (total $n = 18$), within-batch and between-batch precision (expressed as relative standard deviation, RSD) and accuracy were evaluated using freshly prepared calibration curves.

2.4.4 Extraction Recovery and Matrix Effects

Matrix Effect: Pooled blank plasma samples from six individual rats were used. Aliquots (20 μ L each) were spiked with 20 μ L of working solution to prepare low- and high-concentration quality control (QC) samples of psilocin (15 and 400 ng/mL, $n = 6$). For comparison, plasma was replaced with purified water and processed identically, including protein precipitation. The matrix effect was calculated as the ratio of the psilocin peak area in plasma to that in the purified water matrix.

Extraction Recovery Rate: Blank plasma from six individual rats was pooled. To determine extraction recovery, 20 μ L of pooled plasma was spiked with 20 μ L of working solution either before or after protein precipitation, yielding low-, medium-, and high-concentration QC samples (15, 150, and 400 ng/mL, $n = 6$). Recovery was calculated by comparing the measured psilocin concentrations in samples spiked before precipitation with those spiked after precipitation.

2.4.5 Stability

Quality control (QC) samples containing low, medium, and high concentrations of psilocin (15, 150, and 400 ng/mL; $n = 3$ per concentration) were prepared. Their stability was evaluated under the following storage conditions: room temperature for 4 h, 4 °C for 24 h, and –40 °C for 14 days.

2.4.6 Linear Dilution

Six plasma samples containing psilocin at 1500 ng/mL were prepared using blank rat plasma. Each sample was subsequently diluted with blank rat plasma to a final concentration of 150 ng/mL. Accuracy of the test samples was evaluated using a validated standard curve.

2.5 Rat Pharmacokinetic Study

For pharmacokinetic studies, thirty SPF adult Sprague-Dawley (SD) rats (24 males and 6 females, weighing 200–230 g) were employed. The 24 male rats were randomly assigned to four groups ($n = 6$ per group). The 6 female rats constituted a separate medium-dose oral administration group. The dosing regimens were as follows: Intravenous injection (i.v., male): 1 mg/kg, Low-dose oral administration (p.o., male): 1 mg/kg, Medium-dose oral administration (p.o., male and female): 3 mg/kg, High-dose oral administration (p.o., male): 10 mg/kg. The drug was completely dissolved in physiological saline immediately prior to administration. The oral psilocybin dose range (1–10 mg/kg, medium dose 3 mg/kg) was determined by integrating literature and preliminary pharmacokinetic data: the reported effective intraperitoneal dose in mice is 1–10 mg/kg [16,17,18], and a 1:2 oral/intraperitoneal peak concentration ($C_{\max}$) ratio in our preliminary mouse studies gave a calculated oral dose range of 2–20 mg/kg; interspecies scaling to rats yielded an equivalent 1.3–13.3 mg/kg, from which the final experimental doses were established.

Rats were fasted for 12 hours before gavage administration, with free access to water. Normal feeding was resumed 1 hour post-dosing. Blood samples were collected from the jugular vein or retro-orbital plexus at the following time points: pre-dose (0 min), and 2 min (intravenous group only), 5, 15, and 30 min, as well as 1, 2, 4, 6, 8, 12, and 24 hours after administration. Anesthesia was maintained with isoflurane (1.5%–3% in oxygen) throughout the pro-

cedure. Approximately 200 μL of blood was collected per sample and immediately transferred into heparinized centrifuge tubes. The tubes were centrifuged at 4 °C and 2500 $\times g$ for 10 min to separate plasma. Plasma samples were stored at -40 °C until quantitative analysis by LC-MS/MS.

An intravenous group (1 mg/kg) was set as the reference group for calculation of oral bioavailability; oral low, medium, and high-dose groups (1, 3, and 10 mg/kg) were used to assess nonlinear pharmacokinetic characteristics; and a female oral medium-dose group (3 mg/kg) was included to assess sex differences.

2.6 Rat Tissue Distribution Study

Thirty adult male SD rats (SPF grade, 200–230 g body weight) were randomly assigned to five groups ($n = 6$ per group). All rats received psilocybin (3 mg/kg) by oral gavage. Animals in each group were euthanized at 15 min, 1, 4, 6, and 12 h post-administration using carbon dioxide (CO_2) inhalation. Euthanasia was performed by gradually increasing the CO_2 concentration in the chamber to >90% and maintaining it for 5–6 min; the CO_2 used had a purity $\geq 99\%$. Immediately after euthanasia, whole blood was collected via cardiac puncture into heparinized centrifuge tubes. Subsequently, the liver, kidneys, brain, small intestine, stomach, heart, spleen, lungs, skeletal muscle, testes, and adipose tissue were rapidly dissected. All tissue samples were promptly rinsed with ice-cold saline to remove residual blood and contaminants, then gently blotted dry with filter paper. After precise weighing on an electronic balance, extraction solvent was added at a 1:4 (w/v) ratio to prepare homogeneous tissue homogenates: pure acetonitrile was used for liver and kidney tissues, and 50% acetonitrile solution for all other tissues. Homogenates were stored at -40 °C until further analysis.

Following blood collection, the tubes were centrifuged at 2500 $\times g$ for 10 min at 4 °C to separate the plasma. The resulting plasma was stored at -40 °C until subsequent quantitative analysis by LC-MS/MS.

Tissue distribution study (male): a single oral medium dose (3 mg/kg) was administered; this was a descriptive study, and no blank control group was included.

2.7 Rat Excretion Study

A total of six male SD rats were utilized in urinary and fecal excretion studies. The animals were fasted for 12 h before oral gavage with water available ad libitum. Normal diet was reintroduced 1 h post-dosing. Psilocybin was administered as a single oral dose of 10 mg/kg. Immediately prior to administration, the compound was completely dissolved in physiological saline. Following dosing, rats were housed individually in metabolic cages for the collection of cumulative urine and fecal samples at predefined time intervals: 0–6, 6–12, 12–24, 24–36, 36–48, 48–72, and 72–96 h post-administration. Urine volume was accurately measured and recorded; fecal samples were weighed imme-

diately after collection and homogenized with acetonitrile (1:4, w/v) for subsequent processing. All collected samples were stored at -40 °C until subsequent analysis.

An additional six male SD rats were utilized for bile excretion experiments. The rats were anesthetized with sodium pentobarbital (2% physiological saline solution, 0.3 mL per 100 g body weight) and underwent bile duct cannulation surgery. Following a 24-hour postoperative recovery period, the cannula was exteriorized via the duodenum. Normal bile flow was verified prior to drug administration. A single oral dose of psilocybin (10 mg/kg) was then administered to rats, which were placed in rodent restraint jackets with free access to water during bile collection. Bile samples were collected at 0–6, 6–12, 12–24, 24–36, and 36–48 h post-administration, and their volumes were recorded. All other experimental procedures were identical to those described previously.

All psilocybin concentrations in biological samples were converted to psilocin equivalents for data analysis based on equimolar conversion.

Excretion study (male): a single oral high dose (10 mg/kg) was administered; urine, feces, and bile were collected; this was a descriptive study, and no blank control group was included.

2.8 P-Glycoprotein (MDR1)-Mediated Efflux Transport

To assess whether psilocin is a substrate for P-glycoprotein (P-gp), a bidirectional transport assay was performed using Transwell inserts and LLC-PK1-MOCK/MDR1 cell lines. Cells in the logarithmic growth phase were seeded at a density of 2×10^4 cells per well into 24-well plates equipped with 6.5 mm Transwell inserts and cultured in a CO_2 incubator at 37 °C for 7 days. On day 5, transepithelial electrical resistance (TEER) was measured with a cell resistance meter. The bidirectional transport experiment was initiated once the TEER value of LLC-PK1 cells reached or exceeded $70 \Omega \cdot \text{cm}^2$.

Based on the maximum concentration of psilocin observed *in vivo* (820 ng/mL), we selected half of this value (i.e., 410 ng/mL) for our *in vitro* experiments. This concentration corresponds to approximately 2 μM , which was intended to better mimic the *in vivo* experimental conditions in the *in vitro* environment. Prior to the experiment, wash the cells three times with pre-warmed HBSS buffer (containing 10 mM HEPES and 0.1% BSA). After the third wash, retain the buffer in the chamber and pre-incubate the system at 37 °C for 30 min. Apical-to-basolateral (A-B) transport assay: Add 150 μL of buffer containing 2 μM psilocin to the apical compartment (side A) and 600 μL of blank buffer to the basolateral compartment (side B). Basolateral-to-apical (B-A) transport assay: Add 650 μL of drug-containing buffer to side B and 100 μL of blank buffer to side A. Immediately after adding the solutions to both sides, collect 50 μL samples from each compartment for quantification of the initial dose. Following incubation

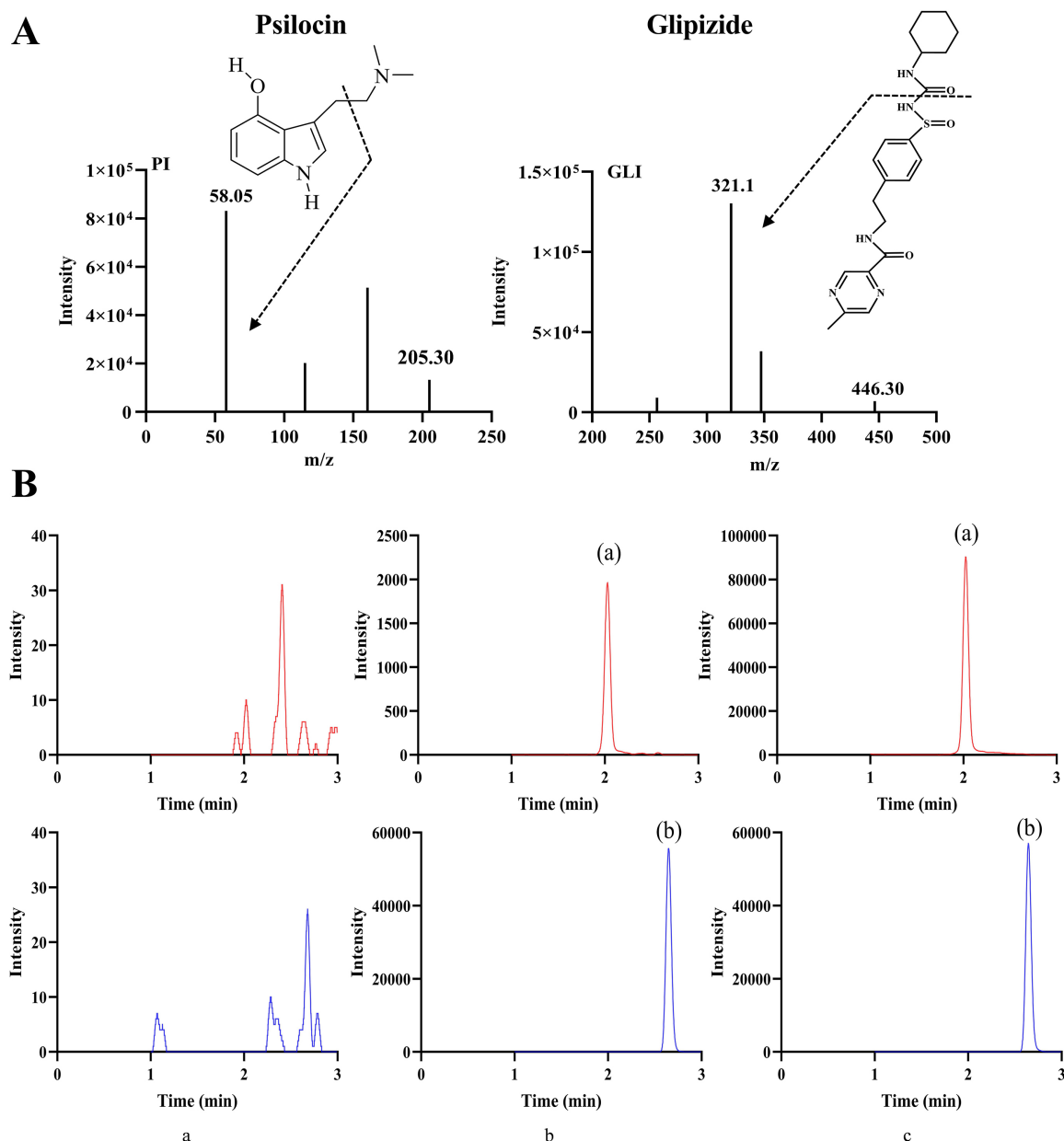


Fig. 2. Analysis of psilocin and glipizide by tandem mass spectrometry and chromatography. (A) Representative tandem mass spectrometry spectra for psilocin and glipizide. (B) Chromatograms of rat plasma samples. a: Blank plasma; b: Blank plasma spiked with psilocin and glipizide at 5 ng/mL (working solution); c: Plasma sample collected 15 minutes after oral administration of 3 mg/kg psilocybin; (a) Psilocin; (b) Glipizide.

at 37 °C for 2 h, aspirate 100 μ L samples from both the donor and receiver compartments. A positive control group was included in parallel.

P-gp efflux assay: LLC-PK1-MOCK (blank control) and LLC-PK1-MDR1 (P-gp-overexpressing) cell lines were compared. Metoprolol (high permeability) and atenolol (low permeability) served as permeability controls, and digoxin served as a positive substrate control.

2.9 Transmembrane Transport in Caco-2 Cells

To evaluate the oral transmembrane absorption characteristics of psilocin, a Transwell transport assay was performed using Caco-2 cell monolayers. Caco-2 cells in log-phase growth were seeded at a density of 2×10^4 cells per well into 6.5 mm Transwell inserts placed in a 24-well plate. Following 21 days of culture, transepithelial electrical resistance (TEER) was measured with a cell resistance meter. Monolayers exhibiting TEER values greater than 1200 $\Omega \cdot \text{cm}^2$ were used for transport studies.

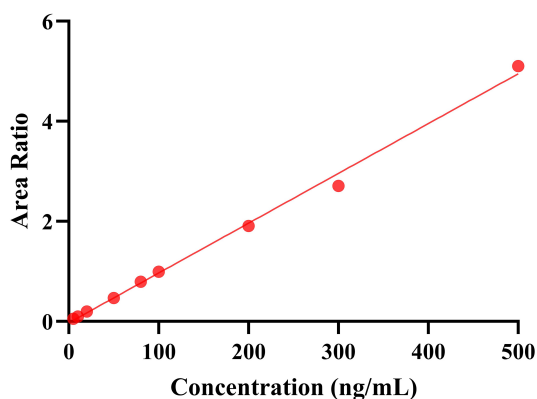


Fig. 3. The calibration curve of psilocin in rat plasma.

Prior to the experiment, aspirate the culture medium from the chambers and rinse three times with pre-warmed (37 °C) buffer consisting of Hanks' solution supplemented with 10 mM HEPES and 0.1% BSA. During the third rinse, add 100 μ L of buffer to the upper chamber and 600 μ L to the lower chamber. After pre-incubation at 37 °C for 30 min, remove all liquid from both chambers. A-B Transport Assay: Add 150 μ L of pre-warmed drug solution (2 μ M psilocin, or a mixture of 2 μ M each of atenolol, metoprolol, and digoxin) to the upper chamber and 600 μ L of buffer to the lower chamber. B-A Transport Assay: Add 100 μ L of buffer to the upper chamber and 650 μ L of drug solution to the lower chamber. Immediately following drug addition, collect 50 μ L samples from both the upper and lower chambers to determine the initial (time-zero) concentration. After incubating at 37 °C for 2 h, withdraw 100 μ L samples from each chamber. All samples were processed by protein precipitation and analyzed using LC-MS/MS.

Caco-2 transport assay: Metoprolol (high permeability) and atenolol (low permeability) served as permeability controls, and digoxin served as a P-gp functional control. The Caco-2 transport assay was a permeability assessment, not an interventional study, and no blank control group was included.

2.10 Data Processing

This study did not prespecify formal exclusion criteria for animals or data points. All Sprague-Dawley (SD) rats were purchased from Beijing Charles River Laboratory Animal Technology Co., Ltd. (Beijing, China). Upon arrival, rats underwent a health examination (no signs of disease or trauma, body weight 200–230 g, normal activity) before enrollment; this was a routine animal receipt procedure. Because all study endpoints were objective quantitative measurements (e.g., plasma drug concentrations, tissue concentrations), no prespecified outlier exclusion criteria were established. No data points were excluded during the data analysis phase.

No animals, experimental units, or data points were excluded from any experiment. Pharmacokinetics (30 ani-

mals): all animals completed dosing, blood sampling, and analysis. Tissue distribution (30 animals): all animals were euthanized on schedule and tissue collection was completed. Excretion study (12 animals): all collections were completed; all bile duct cannulation surgeries were successful. *In vitro* experiments: transport was completed in all Transwell inserts, TEER values met acceptance criteria, and no well leakage occurred.

The exact *n* values at the final analysis for each group were identical to the initial allocation: *n* = 6 per group in the animal experiments, and *n* = 3 per condition in the cell experiments.

Key pharmacokinetic parameters were determined using a non-compartmental approach in WinNonlin software (version 8.1, Certara, Princeton, NJ, USA). These included elimination half-life ($t_{1/2}$), time to peak concentration (T_{max}), peak concentration (C_{max}), area under the concentration–time curve (AUC), and mean residence time (MRT). Bioavailability (*F*) was calculated using Eqn. 1. Tissue partition coefficients (K_p) were derived according to Eqn. 2. The apparent permeability coefficient (P_{app}), efflux ratio (ER), and recovery were computed using Eqns. 3,4,5, respectively.

All experimental data are expressed as mean $\pm$ standard deviation (SD). During data analysis, the normality of all continuous variables was assessed using the Shapiro–Wilk normality test in GraphPad Prism 10.1. The results indicated that none of the continuous variables, including PK parameters across all dose groups and *in vitro* data, significantly deviated from a normal distribution ($p > 0.05$). Statistical analysis was performed using GraphPad Prism 10.1 software. Comparisons among multiple groups were conducted using one-way ANOVA, while comparisons between two groups were performed using independent samples *t*-tests. $p < 0.05$ indicates a statistically significant difference; $p < 0.01$ indicates a highly significant difference.

This study was not blinded. The study personnel were aware of the animal identification numbers and group assignments throughout the study. Specifically, group allocation was performed by investigators using a random number table, and cages were labeled with animal numbers and group assignments; dosing personnel administered different dosing regimens according to group assignment, which made blinding objectively infeasible; LC-MS/MS analysts were aware of the sample group origin, but the analysis was performed using automated instrumentation, yielding objective data; statistical personnel were aware of the group assignments, but pharmacokinetic parameters were calculated using standardized non-compartmental analysis, without subjective bias. Because all endpoints were objective and quantitative, the absence of blinding had minimal impact on the results.

This was an exploratory pharmacokinetic study rather than a hypothesis-testing study, and no single primary endpoint was prespecified. The parameters of primary inter-

Table 1. Precision and accuracy of psilocin in rat plasma (mean $\pm$ SD, n = 6 biological duplications).

Concentration (ng/mL)	Intra-day (n = 6)						Inter-day (n = 18)	
	Accuracy (%)			RSD (%)			Accuracy (%)	RSD (%)
5	94.0 $\pm$ 8.09	92.3 $\pm$ 8.63	87.6 $\pm$ 2.99	8.6	9.4	3.4	91.3 $\pm$ 3.29	3.6
15	96.3 $\pm$ 6.07	100.6 $\pm$ 5.39	90.1 $\pm$ 3.30	6.3	5.4	3.7	95.7 $\pm$ 5.28	5.5
150	103.8 $\pm$ 1.21	106.5 $\pm$ 3.31	95.2 $\pm$ 8.49	1.2	3.1	8.9	101.8 $\pm$ 5.94	5.8
400	108.2 $\pm$ 3.32	107.3 $\pm$ 5.58	97.0 $\pm$ 7.01	3.1	5.2	7.2	104.1 $\pm$ 6.24	6.0

SD, standard deviation; RSD, relative standard deviation.

Table 2. Extraction recovery and matrix effects of psilocin in rat plasma (mean $\pm$ SD, n = 6).

Concentration (ng/mL)	Extraction recovery (%)	Matrix effect (%)	Matrix effect RSD (%)
15	104.1 $\pm$ 10.09	101.1 $\pm$ 2.57	2.5
150	108.9 $\pm$ 3.00	/	/
400	107.8 $\pm$ 2.72	102.3 $\pm$ 1.08	1.1

est were AUC and ($C_{\max}$) (systemic exposure), (K_p) (tissue distribution), cumulative recovery (excretion), and (P_{app}) and ER (permeability/efflux). Pharmacokinetic parameters were calculated using non-compartmental analysis and are generally considered to be approximately normally distributed, with n = 6 per group. This was an exploratory, descriptive study, and statistical testing was used only as a supplementary reference.

$$F = \frac{\text{AUC}_{(0-\infty, \text{extravascular})} \times \text{Dose}_{\text{i.v.}}}{\text{AUC}_{(0-\infty, \text{i.v.})} \times \text{Dose}_{\text{extravascular}}} \times 100\% \quad (1)$$

$$K_p = \text{AUC}_{(0-t, \text{tissue})} / \text{AUC}_{(0-t, \text{plasma})} \quad (2)$$

$$P_{\text{app}} = (dQ/dt) / (A \times C_0) \quad (3)$$

$$\text{ER} = P_{\text{app(B-A)}} / P_{\text{app(A-B)}} \quad (4)$$

$$\text{Recovery} = (\text{TR}_{\text{cumrf}} + C_{\text{df}} \times V_D) / (C_{\text{di}} \times V_D) \times 100\% \quad (5)$$

dQ/dt represents the drug transport rate per unit time ($\mu\text{M/s}$), A denotes the surface area of the polycarbonate membrane (0.33 cm^2), and C_0 indicates the initial concentration of the target compound (μM). TR_{cumrf} denotes the cumulative drug transport volume in the receiving well at the end of incubation (nmol), C_{df} represents the drug concentration in the feeding well at the end of incubation (μM), V_D is the volume of the feeding well (mL), and C_{di} indicates the drug concentration in the feeding well at the start of incubation (μM).

3. Results

3.1 Methodological Validation of Psilocin Quantification in Rat Plasma

3.1.1 Selectivity

Glipizide was selected as the internal standard (IS) for the quantitative analysis of psilocin in this study. As a widely used IS in bioanalytical investigations, glipizide has a well-established and stable LC-MS/MS analytical method, facilitating its straightforward application and validation in the present experimental system. Furthermore, glipizide and psilocin achieved satisfactory chromatographic retention time separation under the established analytical conditions, with no mutual chromatographic interference observed between the two analytes. This ensured the avoidance of any interference with quantitative detection, thereby guaranteeing the accuracy and reliability of the obtained quantitative analysis results. The corresponding product ion mass spectra of psilocin and glipizide are presented in Fig. 2A. Fig. 2B displays representative chromatograms of blank rat plasma, blank plasma spiked with psilocin and glipizide (internal standard) working solutions, and post-administration plasma samples. The response from endogenous components in blank rat plasma was less than 20% of the lower limit of quantification for psilocin and below 5% of that for the internal standard, glipizide. The signal-to-noise (S/N) ratio for both analytes in rat plasma exceeded 5. No interference from endogenous plasma constituents was observed in the determination of psilocin or glipizide.

3.1.2 Calibration Curve and Limit of Quantification

The linear range for psilocin in rat plasma was established to be 5–500 ng/mL. A calibration curve was constructed by plotting psilocin concentration (X , ng/mL) on the abscissa against the peak area ratio of psilocin to internal standard (Y) on the ordinate. Weighted ($1/X^2$) least-squares regression analysis yielded a calibration equation of $Y = 0.00965857X - 0.00275871$ ($r^2 = 0.9982$). Rel-

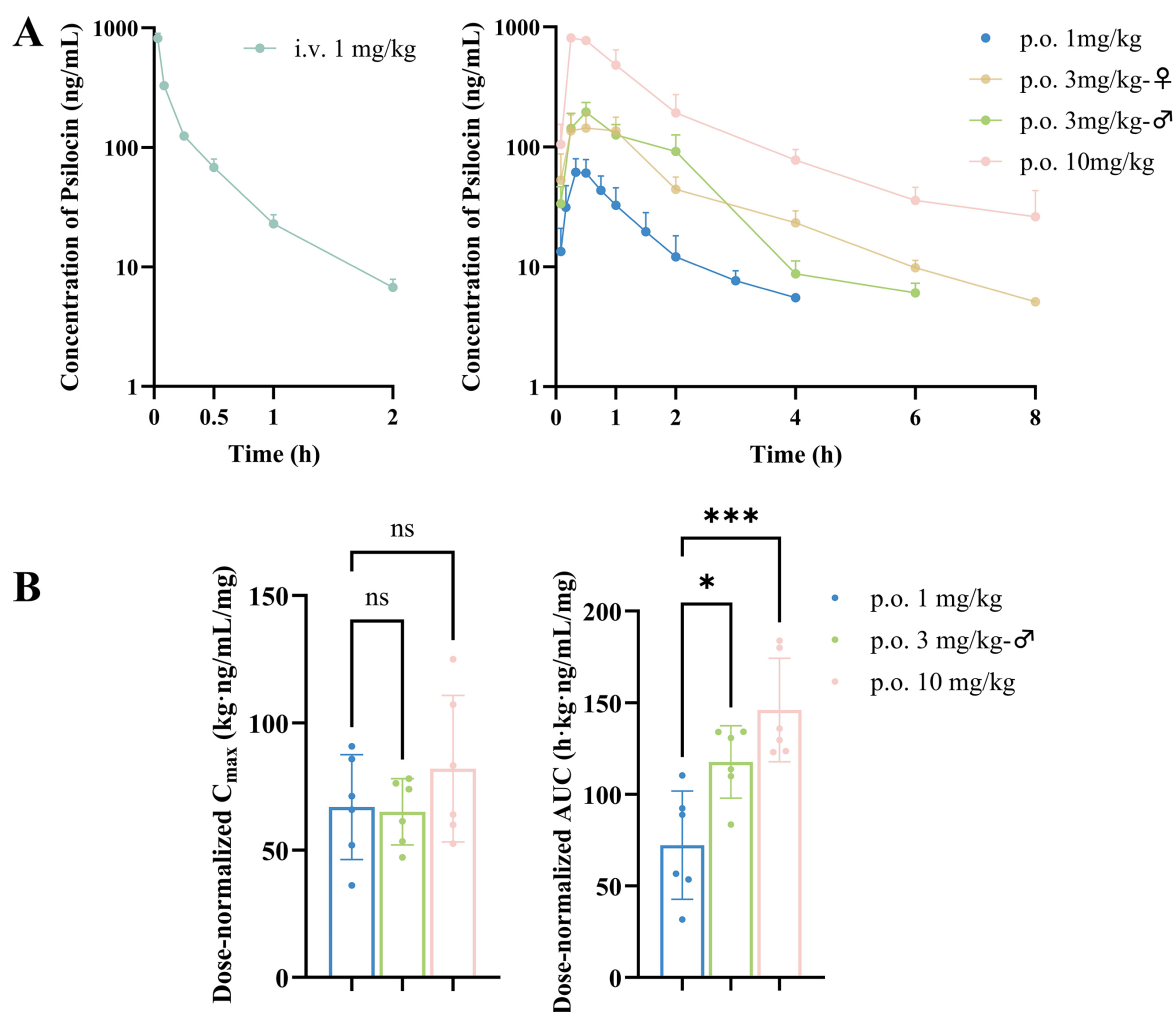


Fig. 4. Pharmacokinetic characteristics of psilocin following intravenous and oral administration of different doses of psilocybin in rats (n = 6). (A) Plasma concentration–time profile of psilocin. (B) Dose-normalized C_{max} and AUC of psilocin in the oral administration. Multi-group comparisons were conducted via one-way analysis of variance (One-way ANOVA); * $p < 0.05$ indicates a statistically significant difference, *** $p < 0.001$ indicates a highly statistically significant difference; “ns” represents no significant difference. C_{max} , peak concentration; AUC, area under the concentration–time curve.

ative standard deviation (RSD) values for the calibration standards were within the acceptable criteria (in accordance with bioanalytical method validation guidelines), confirming a robust linear relationship between psilocin concentration and detector response. The lower limit of quantification (LLOQ) for the rat plasma is 5 ng/mL. The calibration curve is presented in Fig. 3.

3.1.3 Accuracy and Precision

Both inter- and intra-assay precision values for quality control samples were below 9.4%, while accuracy ranged from 87.6% to 108.2%. These results comply with the validation requirements outlined in the ICH M10 guideline. Detailed data are provided in Table 1.

3.1.4 Extraction Recovery Rate and Matrix Effect

The extraction recovery of psilocin from rat plasma ranged from 104.1% to 108.9%. The matrix effect for psilocin in individual rat plasma samples was between 101.1% and 102.3%, with a coefficient of variation (CV) of 1.1% to 2.5%, indicating negligible matrix interference. These results, detailed in Table 2, comply with the M10 bio-analytical method validation guidelines.

3.1.5 Stability

Plasma samples containing psilocin were stored under three conditions: room temperature for 4 hours, 4 °C for 1 day, and –40 °C for 14 days. When analyzed against freshly prepared standard curves, the measured concentrations of all stability samples fell within 87.6%–108.5% of their theoretical values, with precision below 8.6%. These results demonstrate that psilocin remains stable in rat plasma under

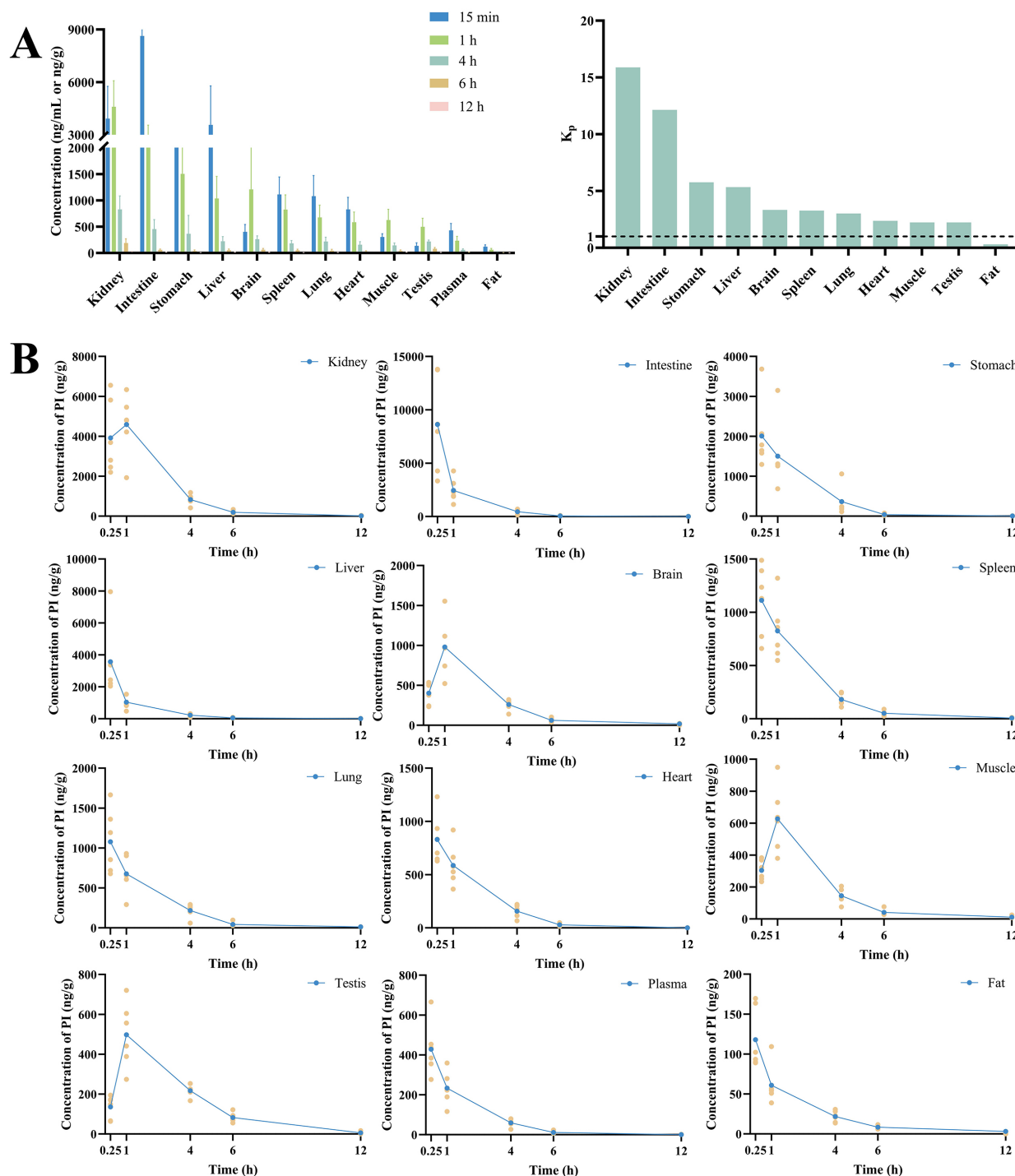


Fig. 5. Tissue distribution of psilocin following oral administration of 3 mg/kg psilocybin in rats (n = 6). (A) Bar chart depicting the mean psilocin concentration and K_p in each examined tissue. (B) Mean time-concentration profiles of psilocin in various tissues.

the tested storage conditions, thereby fulfilling the method validation criteria outlined in the M10 guideline. Detailed data are provided in Table 3.

3.1.6 Linear Dilution

Psilocin-fortified plasma samples (1500 ng/mL) were diluted with blank rat plasma to a final concentration of 150 ng/mL. The measured accuracy was determined as $105.7 \pm$

5.51%, which met the pre-defined acceptance criteria for bioanalytical method validation. Detailed results are provided in Table 4.

3.2 Pharmacokinetic Characteristics in Rats

Plasma concentration-time profiles of psilocin following intravenous (1 mg/kg) and oral (1, 3, and 10 mg/kg) administration of psilocybin in rats are presented

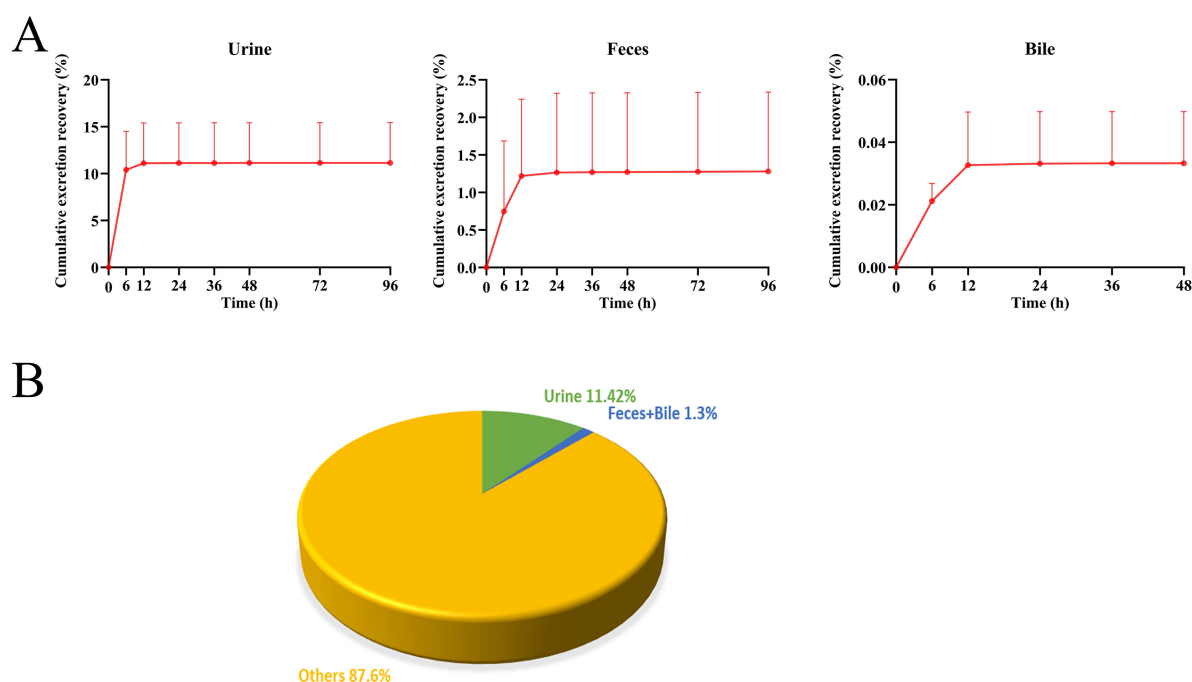


Fig. 6. Excretion characteristics of psilocin following oral administration of 10 mg/kg psilocybin in rats (n = 6). (A) Cumulative excretion recovery. (B) Cumulative excretion recovery is presented as a pie chart.

Table 3. Stability of psilocin in rat plasma (mean $\pm$ SD, n = 3).

Concentration (ng/mL)	Room temperature for 4 h		4 °C for 1 d		–40 °C for 14 d	
	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
15	95.7 $\pm$ 5.06	5.30	87.6 $\pm$ 1.94	2.20	108.5 $\pm$ 2.59	2.40
150	102.6 $\pm$ 8.78	8.60	90.8 $\pm$ 4.37	4.80	105.4 $\pm$ 3.30	3.10
400	94.4 $\pm$ 1.99	2.10	90.8 $\pm$ 2.03	2.20	108.4 $\pm$ 1.97	1.80

in Fig. 4A. Key pharmacokinetic parameters derived from non-compartmental analysis are summarized in Table 5. After intravenous dosing, psilocin displayed a short elimination half-life (0.4 ± 0.03 h). Its systemic clearance (4.4 ± 0.4 L/h/kg) exceeded the typical hepatic blood flow in rats (3.3 L/h/kg), while the apparent volume of distribution at steady state (1.5 ± 0.1 L/kg) was considerably larger than the extracellular fluid volume of rats (0.297 L/kg). Following oral administration, the time to reach peak concentration (T_{max}) ranged from 0.3 to 0.7 h across dose groups. After normalization for dose (Fig. 4B), the peak concentrations (C_{max}) did not differ significantly among dose groups ($p > 0.05$). In contrast, systemic exposure (AUC) in the 3 mg/kg and 10 mg/kg groups increased by 63% and 102%, respectively, relative to the 1 mg/kg group, indicating nonlinear pharmacokinetics. Oral bioavailability was 47.6%, 72.4%, and 90.7% at 1, 3, and 10 mg/kg, respectively. Moreover, in the medium-dose group (3 mg/kg), no statistically significant differences ($p > 0.05$) were observed between male and female rats in any primary pharmacokinetic parameter (T_{max} , C_{max} , $AUC_{0-\infty}$, etc.), suggesting an absence of sex-related differences in the pharmacokinetic profile of psilocin in rats.

3.3 Distribution Characteristics in Rat Tissues

Following oral administration of psilocybin (3 mg/kg) to rats, the concentration-time profiles of psilocin in plasma and various tissues were derived using non-compartmental analysis based on measured concentrations at multiple time points. The tissue-to-plasma partition coefficient (K_p) was calculated, and the tissue distribution characteristics of psilocin are presented in Fig. 5. Psilocin demonstrated extensive tissue distribution in rats. K_p values exceeded 1 in all tissues except adipose tissue, with notably high distribution into the brain, liver, stomach, intestine, and kidneys. Maximum concentrations (C_{max}) were generally attained within 15 min in most tissues; however, in testes, muscle, brain, and kidneys, C_{max} was reached at 1 h, reflecting rapid distribution kinetics. Within six hours post-administration, drug concentrations in most tissues declined to approximately 5% of C_{max} , indicating rapid elimination.

3.4 Excretion Characteristics in Rat

Following oral administration of psilocybin in rats, the cumulative excretion recovery of psilocin via each pathway generally plateaued within 12 h post-dose, as shown

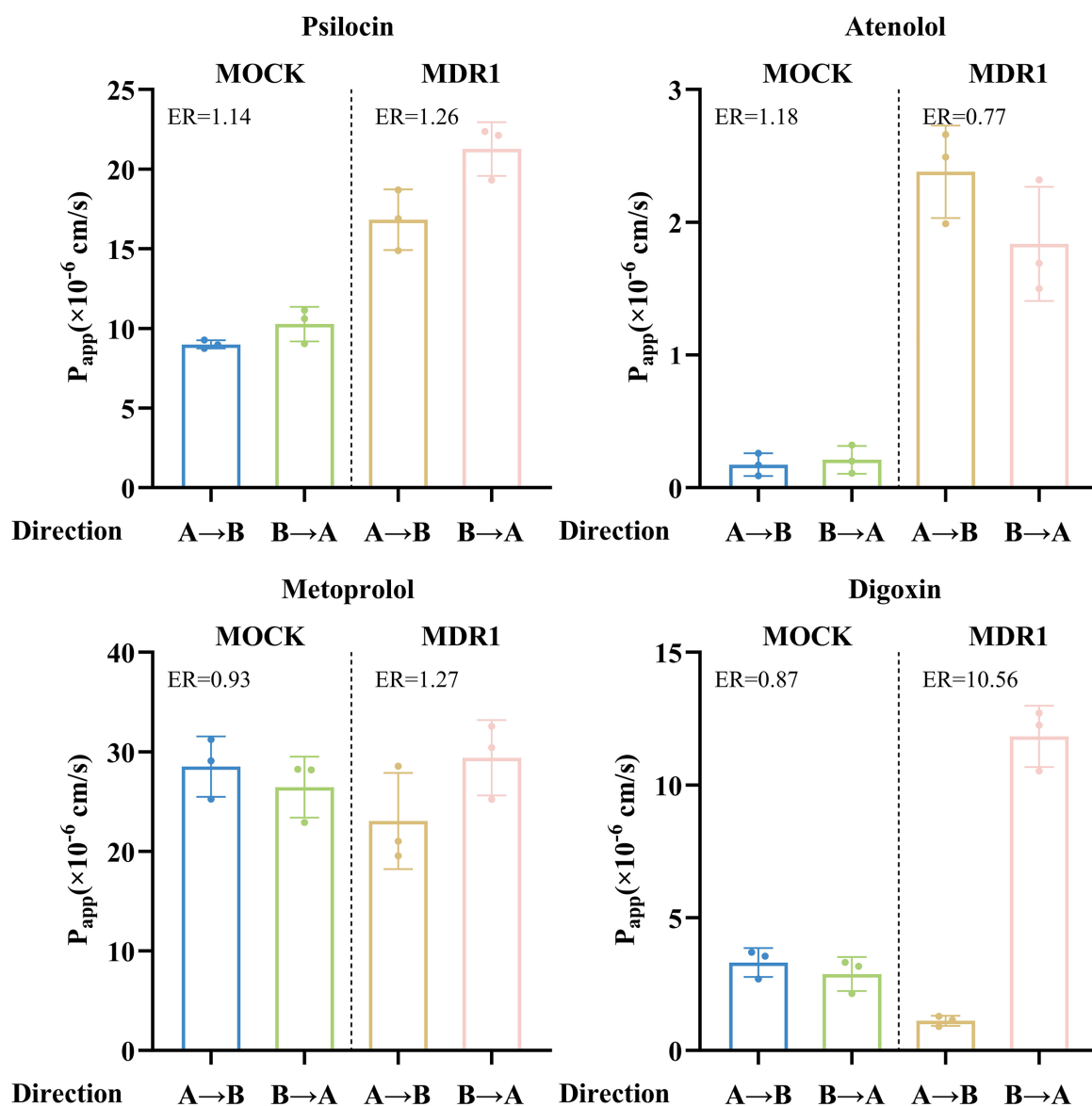


Fig. 7. Bidirectional characteristics transport of psilocin in LLC-PK1/MDR1 cell models (n = 3). ER, efflux ratio.

Table 4. Linear dilution of psilocin in rat plasma samples (mean $\pm$ SD, n = 6).

Biological matrix	Concentration (ng/mL)	Diluted factor	Accuracy (%)
Plasma	1500	10	105.7 $\pm$ 5.51

in Fig. 6A and summarized in the corresponding pie chart (Fig. 6B). Urinary excretion represented the predominant elimination route, accounting for 11% of the administered dose. In contrast, fecal and biliary pathways demonstrated comparatively lower cumulative recoveries. Overall, the total cumulative recovery across all three excretion routes was approximately 13% of the administered dose, which is markedly lower than the delivered dose.

3.5 P-gp (MDR1)-Mediated Efflux Transport

Based on the nonlinear pharmacokinetic behavior of psilocin observed in rats—where dose-normalized C_{max}

varied linearly, while AUC increased significantly—this study further utilized the LLC-PK1-MDR1 cell model to systematically examine the role of P-gp, a major efflux transporter in the intestine and blood–brain barrier (BBB), in regulating the transmembrane transport of psilocin. A Transwell bidirectional transport assay was employed for this investigation. In the established cellular model, metoprolol (a high-permeability positive control) exhibited moderate permeability, whereas atenolol (a low-permeability positive control) showed low permeability. These results confirm that the cells formed a tight monolayer barrier, fulfilling the functional criteria for Transwell transport exper-

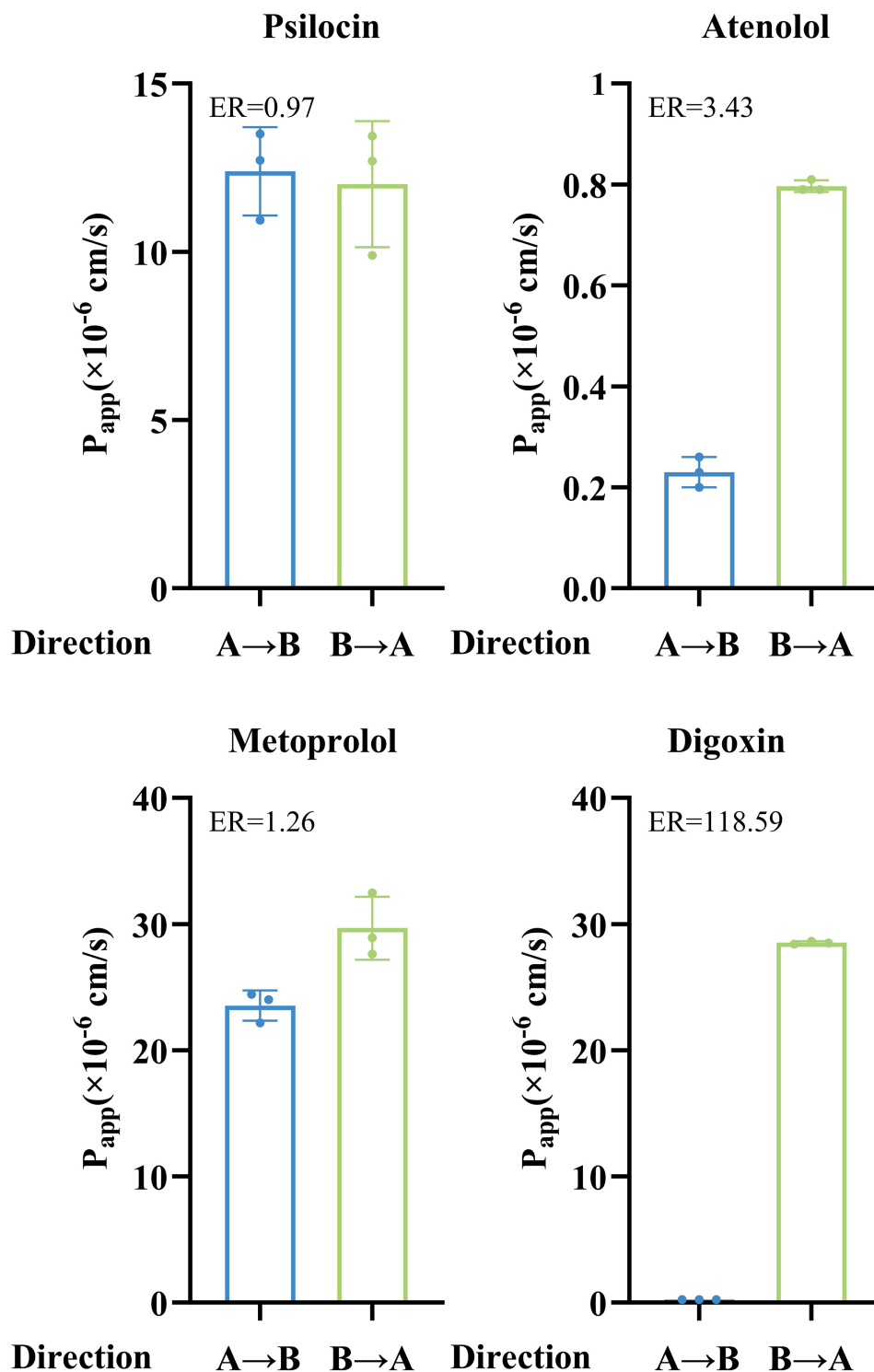


Fig. 8. Bidirectional characteristics transport of psilocin in Caco-2 cell models (n = 3).

iments. Moreover, the efflux ratio (ER) of digoxin, a specific P-gp substrate, was significantly higher in LLC-PK1-MDR1 cells than in MOCK cells. This indicates robust functional activity of the target transporter (P-gp) within the cellular model, thereby verifying the stability and reliability of the experimental system. The transport characteristics of psilocin in this model are presented in Fig. 7. With an ER

value of 1.26, psilocin is not a substrate of P-gp, suggesting that its transmembrane transport *in vivo* is not influenced by P-gp-mediated efflux mechanisms.

3.6 Transmembrane Transport in Caco-2 Cells

In the Caco-2 cell Transwell model, the apparent permeability coefficient (P_{app}) of the highly permeable drug

Table 5. Pharmacokinetic parameters of psilocin following intravenous and oral administration of different doses of psilocybin in rats (mean $\pm$ SD, n = 6).

Parameters	Unit	i.v. (mg/kg)		p.o. (mg/kg)		
		1	1	3		10
				σ	φ	
$t_{1/2}$	h	0.4 $\pm$ 0.03	0.7 $\pm$ 0.2	0.8 $\pm$ 0.2	1.5 $\pm$ 0.5	2.6 $\pm$ 1.0
T_{max}	h	/	0.4 $\pm$ 0.1	0.5 $\pm$ 0.0	0.7 $\pm$ 0.3	0.3 $\pm$ 0.1
C_{max}	ng/mL	/	66.9 $\pm$ 20.6	195.1 $\pm$ 39.0	159.9 $\pm$ 42.8	820.0 $\pm$ 288.3
Dose-normalized C_{max}	kg·ng/mL/mg	/	66.9 $\pm$ 20.6	65.0 $\pm$ 13.0	53.3 $\pm$ 14.3	82.0 $\pm$ 28.8
C_0	ng/mL	1373.3 $\pm$ 253.6	/	/	/	/
$AUC_{(0-t)}$	h·ng/mL	162.4 $\pm$ 13.1	72.2 $\pm$ 29.6	352.9 $\pm$ 59.4	307.1 $\pm$ 59.7	1459.8 $\pm$ 282.6
Dose-normalized AUC	h·kg·ng/mL/mg	162.4 $\pm$ 13.1	72.2 $\pm$ 29.6	117.6 $\pm$ 19.8	102.4 $\pm$ 19.9	146.0 $\pm$ 28.3
$AUC_{(0-\infty)}$	h·ng/mL	166.3 $\pm$ 13.9	79.1 $\pm$ 30.3	361.3 $\pm$ 57.1	329.3 $\pm$ 61.9	1507.7 $\pm$ 259.2
$MRT_{(0-t)}$	h	0.3 $\pm$ 0.03	0.9 $\pm$ 0.2	1.4 $\pm$ 0.2	1.6 $\pm$ 0.4	2.3 $\pm$ 0.6
V_z	L/kg	2.7 $\pm$ 0.2	/	/	/	/
V_{ss}	L/kg	1.5 $\pm$ 0.1	/	/	/	/
CL	L/h/kg	4.4 $\pm$ 0.4	/	/	/	/
F	%	/	47.6	72.4	66.0	90.7

$t_{1/2}$, elimination half-life; T_{max} , time to peak concentration; MRT, mean residence time; V_z , Volume of distribution; V_{ss} , Volume of distribution at steady state; CL, Clearance; F, Bioavailability.

metoprolol was $23.31 \pm 0.98 \times 10^{-6}$ cm/s, whereas that of the low-permeability drug atenolol was $0.23 \pm 0.02 \times 10^{-6}$ cm/s. These results confirm the well-maintained tight junctions and barrier integrity of the Caco-2 cell monolayer. Furthermore, the cells exhibited high expression of P-glycoprotein (P-gp), as evidenced by an efflux ratio (ER) of 118.59 for the classic P-gp substrate digoxin, indicating preserved P-gp transporter function. Together, these data demonstrate that the established Caco-2 Transwell model meets the quality control standards for *in vitro* drug absorption studies. The P_{app} value for psilocin was determined to be $12.39 \pm 1.31 \times 10^{-6}$ cm/s (Fig. 8), placing it within the moderate permeability range. This suggests that psilocin possesses moderate permeability, a property that likely facilitates its effective penetration across the intestinal epithelial barrier and subsequent entry into systemic circulation following oral administration *in vivo*.

4. Discussion

As a prodrug of psilocin, psilocybin is rapidly dephosphorylated *in vivo* by alkaline phosphatase to its active metabolite, psilocin [19]. Therefore, the primary objective of psilocybin pharmacokinetic studies is to clarify the exposure-response relationship of psilocin. By quantifying psilocin concentrations in biological matrices, the *in vivo* exposure levels of psilocybin can be characterized, allowing for systematic evaluation of its therapeutic efficacy and safety.

The LC-MS/MS method developed in this study for the quantitative determination of psilocin proved to be highly efficient and reliable upon validation in biological matrices. Through systematic optimization during prelim-

inary experiments, a linear range of 5–500 ng/mL was established, encompassing the concentration levels typically observed after various administered doses and thereby ensuring the method's applicability to real-world samples. Sample pretreatment was performed using a straightforward protein precipitation protocol, which is simpler than previously reported methods such as liquid-liquid extraction or solid-phase extraction [20], thereby facilitating easier operation. In accordance with the M10 Bioanalytical Method Validation guideline, key parameters including precision, accuracy, and stability were comprehensively evaluated, with all results meeting the predefined acceptance criteria. To mitigate the poor stability of psilocin during the preparation of rat tissue homogenates, extraction solvents with different water–acetonitrile ratios were compared to identify the optimal composition, enabling reliable quantification of psilocin in tissue samples.

Existing research has confirmed that psilocin undergoes multi-pathway metabolism *in vivo*. Owing to its structural similarity to serotonin (5-HT), monoamine oxidase-A (MAO-A) acts as the primary Phase I metabolic enzyme. Subsequently, aldehyde dehydrogenase (ALDH) further participates in this pathway, leading to the formation of 4-hydroxyindole-3-acetic acid (4-HIAA). In addition, cytochrome P450 enzymes, specifically CYP3A4 and CYP2D6, contribute to psilocin metabolism. Psilocin is also metabolized into psilocin-O-glucuronide via glucuronidation, primarily catalyzed by UGT1A10 and UGT1A9 [21,22].

Pharmacokinetic studies in rats demonstrated that the clearance of psilocin—the active metabolite of psilocybin—following intravenous psilocybin administration significantly exceeded hepatic blood flow. This suggests substan-

tial extrahepatic clearance *in vivo*, consistent with the multi-pathway, multi-organ metabolic profile of psilocin. Furthermore, the volume of distribution of psilocin was greater than the extracellular fluid volume in rats, indicating extensive tissue distribution. After oral administration of varying psilocybin doses to rats, psilocin was rapidly absorbed across the gastrointestinal tract. Dose-normalized $C_{\max}$ showed no significant differences across the tested dose groups. In contrast, the area under the AUC increased in a dose-dependent manner with statistically significant inter-group differences, indicative of prominent nonlinear pharmacokinetic characteristics for psilocybin. The consistent intestinal absorption profiles across all dose groups implied that neither intestinal metabolic processes nor membrane permeation was saturated within the tested dose range. Given the metabolic characteristics of psilocin, the observed nonlinear pharmacokinetic behavior is presumably attributable to the saturation of hepatic metabolic enzymes involved in its biotransformation. Following medium-dose administration, no statistically significant sex-related differences were observed in the pharmacokinetic parameters of psilocin. Although prior studies report sex differences in rat hepatic expression of UGT1A10 and CYP3A1 (the rat orthologue of human CYP3A4), no such differences have been documented for MAO-A or CYP2D1/2D3 (rat orthologues of human CYP2D6) [23,24]. Given the multiple metabolic pathways involved in psilocin elimination, the absence of significant sex differences at a moderate dose in rats is biologically plausible. We also found that psilocin exhibits a short half-life via both intravenous and oral routes. Existing references suggest that despite psilocin's short plasma half-life, its neuropsychotropic effects can induce sustained and enduring neuroplastic changes [25,26]. Therefore, the persistence of therapeutic effects may be a more critical determinant of dosing intervals than the rate of plasma concentration decay alone. Consequently, the optimal dosing regimen for psilocybin requires further investigation in conjunction with detailed pharmacodynamic studies.

In rat tissue distribution studies, psilocin demonstrated rapid and extensive systemic distribution. The tissue-plasma partition coefficient (K_p) was greater than 1 in all tissues examined except adipose tissue, consistent with the large volume of distribution derived from plasma pharmacokinetic parameters. By 6 h post-administration, psilocin concentrations in rat tissues had declined substantially, with no indication of tissue accumulation.

In excretion studies, the cumulative recovery of unchanged psilocin was only 13%, indicating that its elimination occurs primarily via *in vivo* metabolism.

The intestinal absorption and tissue distribution profiles of psilocin were further investigated using Caco-2 cell transport assays and monolayer epithelial cells overexpressing hMDR1. Transport studies in Caco-2 cells demonstrated that psilocin possesses moderate intestinal

permeability and is not a substrate for P-gp. After low-dose oral administration of psilocybin, the bioavailability of psilocin was approximately 45%, indicating that under non-saturating conditions, intestinal and hepatic first-pass metabolism accounted for about 55% of psilocin extraction. Consistent with this, experiments in hMDR1-overexpressing monolayer epithelial cells further confirmed the moderate permeability of psilocin and that it is not a substrate for P-glycoprotein. These results align with the extensive tissue distribution of psilocin observed in multiple organs, particularly its elevated concentrations in the brain—the target organ. Together, these findings establish a pharmacokinetic basis for the therapeutic effects of psilocin within the central nervous system.

5. Limitations

This article has preliminarily discovered the nonlinear pharmacokinetic characteristics of psilocin in rats and tentatively speculated that the saturation of metabolic enzymes is the reason. However, the specific mechanism still requires further exploration. This limitation does not affect the overall content of the article.

6. Conclusions

In summary, the LC-MS/MS quantitative method developed in this study demonstrates high throughput, sensitivity, and stability, allowing for the reliable measurement of psilocin concentrations in biological matrices. In rats, psilocin shows a short half-life, extensive and rapid tissue distribution, and rapid clearance, with elimination driven primarily by extensive metabolism rather than urinary or fecal excretion. Following oral administration, psilocin exhibits marked nonlinear pharmacokinetics due to metabolic saturation, with no significant sex differences observed. Results from *in vitro* models indicate that psilocin has moderate membrane permeability and is not a P-gp substrate, supporting its efficient penetration into tissues throughout the body, including the brain. Collectively, these findings provide a solid scientific foundation for further pharmacological research and preclinical safety evaluation of psilocin.

Availability of Data and Materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

XZ conceived, designed, and planned this study. JW, BZ and LW performed the experimental procedures. CY, WZ and JX analyzed and interpreted the results, and provided resource support for the study implementation. JW drafted the manuscript with assistance from XZ and BZ, and was responsible for preparing figures and conducting litera-

ture searches. All authors participated in editing and revising the manuscript. Each author reviewed and approved the final version, contributed substantially to the research, and agrees to take responsibility for all aspects of this study.

Ethics Approval and Consent to Participate

All animal experiments were approved by the Animal Care and Use Committee of the Academy of Military Medical Sciences (approval number: IACUC-DWZX-2025-P622). The experimental procedures adhere to the 3Rs principle, minimizing the use of animals as much as possible. The study was carried out in accordance with the ARRIVE guidelines and complied with the Chinese national standard “Guidelines for the Ethical Review of Laboratory Animal Welfare”.

Acknowledgment

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/IJP48587>.

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