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Short communication

# The absorption of oral morroniside in rats: *In vivo, in situ* and *in vitro* studies

SHAN XIONG[1,2,3]*
JINGLAI LI[4]
YANLING MU[1]
ZHENQING ZHANG[4]

[1] *Institute of Materia Medica, Shandong Academy of Medical Sciences, Jinan China*

[2] *Key Laboratory for Biotech-Drugs Ministry of Health, Jinan, China*

[3] *Key Laboratory for Rare and Uncommon Diseases of Shandong Province, Jinan China*

[4] *Key Laboratory of Drug Metabolism and Pharmacokinetics, Beijing Institute of Pharmacology and Toxicology, Beijing China*

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Morroniside is one of the most important iridoid glycosides from *Cornus officinalis* Sieb. et Zucc. In the present study, the pharmacokinetics and bioavailability studies of morroniside were conducted on Sprague-Dawley (SD) rats. A rat *in situ* intestinal perfusion model was used to characterize the absorption of morroniside. Caco-2 cells were used to examine the transport mechanisms of morroniside. The pharmacokinetic study of morroniside exhibited linear dose-proportional pharmacokinetic characteristics and low bioavailability (4.3 %) in SD rats. Its average $P_{eff}$ value for transport across the small intestinal segments changed from $(3.09 \pm 2.03) \times 10^{-6}$ to $(4.53 \pm 0.94) \times 10^{-6}$ cm $s^{-1}$. In Caco-2 cells, the $P_{app}$ values ranged from $(1.61 \pm 0.53) \times 10^{-9}$ to $(1.19 \pm 0.22) \times 10^{-7}$ cm $s^{-1}$ for the apical to basolateral side and the $P_{ratio}$ values at three concentrations were all lower than 1.2. Morroniside showed poor absorption and it might not be a specific substrate of P-glycoprotein (P-gp).

*Keywords:* morroniside, pharmacokinetics, absorption, rats, *in situ* single-pass intestinal perfusion, Caco-2 cell

Morroniside, one of the most important iridoid glycosides, is the main active ingredient of *Cornus officinalis* Sieb. et Zucc. It is a rich source of iridoid glycosides and has been used as a traditional Chinese medicinal herb for centuries (1). Various pharmacological studies have indicated that morroniside was effective in the treatment of Alzheimer's disease (2) for protecting nerves (3), preventing diabetic liver damage (4) and renal damage (5), having beneficial effects on lipid metabolism and inflammation (6, 7) and anti-anaphylactic activity (8). In recent studies, morroniside displayed protective actions against the cytotoxicity produced by exposure to $H_2O_2$ in human SH-SY5Y cells (9, 10). In addition, morroniside could protect ischemia/reperfusion-induced brain injury by decreasing caspase-3 activity, reducing the infarction volume, minimizing oxidative stress, *etc.* (11). Several research papers have been published on the pharmacological activity of ingredients of traditional Chinese medicines but few discuss their pharmacokinetic profiles, which are

* Correspondence; e-mail: shanxiong83@sohu.com

essential. Pharmacokinetic profiles help elaborate the relationship between the intensity and time course of pharmacological events, the *in vivo* toxicological effects of ingredients, and extend the scope of the use and acceptance of different species (12).

In our previous work, the analytical methods for determination of morroniside in rat plasma and beagle dog plasma using LC-MS/MS were reported (13, 14). In addition, the excretion profile of morroniside in rats was investigated by a validated LC-MS/MS method (15).

Thus far, no research was done on the absorption kinetics of morroniside. In the present study, the absorption of oral morroniside was systematically investigated, *in vivo*, *in situ* and *in vitro*. The key absorption issues mentioned are addressed in support of the development of morroniside as a candidate drug.

## EXPERIMENTAL

### *Chemicals*

Morroniside (purity > 98.5 %) was extracted and purified in the Department of Pharmacology, Xuanwu Hospital of Capital Medical University (China). Hyperoside (IS) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (China). LC-MS grade methanol and acetonitrile were obtained from Thermo Fisher Scientific (USA). All other reagents were of analytical grade or better.

Non-essential amino acid solution (100×) and DMEM (high glucose) medium were purchased from Thermo Fisher Scientific. Fetal bovine serum was supplied by Biological Industries (Israel). *N*-[2-hydroxyethyl] piperazine-*N*′-[4-butanesulfonic acid], penicillin and streptomycin solutions, and geneticin were obtained from Amresco (USA).

### *Pharmacokinetic studies*

Twenty healthy male Sprague-Dawley (SD) rats (weighing 180–220 g, $n = 5$ per group) were used for pharmacokinetic studies. They were purchased from the Beijing Institute of Pharmacology and Toxicology (China). Rats housed in polypropylene cages were kept under controlled temperature (20–22 °C) and a 12-h day-night cycle. Animals were used for pharmacokinetic studies after 1-week acclimatization with food and water provided *ad libitum*.

Animal welfare and experimental procedures were approved by the Animal Ethics Committee of the Institute of Materia Medica, Shandong Academy of Medical Sciences (Jinan, China), and strictly complied with the guide for the care and use of laboratory animals (National Research Council of USA, 1996).

Jugular vein catheterization was performed as described previously (16). For bioavailability study, one group of rats was administered an intravenous dose (10 mg $kg^{-1}$ *via* the tail vein) of morroniside. For oral pharmacokinetic studies, three groups of rats were administered an oral dose of 10, 30 and 90 mg $kg^{-1}$ of morroniside, resp. Blood samples (150 μL) were collected from the jugular vein into heparinized tubes before and at 2, 5, 15, 30, 45, 60, 120, 240, 360, 480 and 720 min after single *i.v.* dosing and 5, 15, 30, 45, 60, 120, 240, 360, 480 and 720 min after single oral dosing. Samples were centrifuged at 3500 rpm for 15 min at 4 °C and kept frozen at –20 °C prior to LC-MS/MS analysis.

Pharmacokinetic parameters for morroniside were calculated by the Drug and Statistic (DAS) 2.0 pharmacokinetic software (Chinese Pharmacological Association, Anhui, China). $F_{(0\text{-}\infty)}$ (%) was calculated by the following equation *1*:

$$F_{(0-\infty)} = \frac{AUC_{(0-\infty)}(10 \text{ mg kg}^{-1}{}_{(\text{p.o.})})}{AUC_{(0-\infty)}(10 \text{ mg kg}^{-1}{}_{(\text{i.v.})})} \quad (1)$$

## In situ *permeability studies*

Forty-eight healthy male SD rats (180–220 g, $n = 4$) were used for the *in situ* single-pass perfusion study. Rats were anesthetized *via* an intraperitoneal injection of 1 % pentobarbital sodium (40 mg kg$^{-1}$) and placed in a professional medical incubator to maintain the body temperature at 37 °C. The abdominal cavity was opened by a medioventral line incision, and approximately 10 cm of the duodenum, jejunum, ileum and colon segments were isolated carefully. The intestinal segment was intubated with a pliable plastic pipe at two ends and infused with Krebs-Ringer buffer (7.8 g NaCl, 0.35 g KCl, 0.37 g $CaCl_2$, 1.37 g $NaHCO_3$, 0.32 g $NaH_2PO_4$, 0.02 g $MgCl_2$, 1.4 g glucose and 20 mg phenol red dissolved in 1000 mL distilled water with pH adjusted to 7.4) for 10 min with the flow rate set at 1.0 mL min$^{-1}$ using a single channel micro-infusion pump (WZ-50C66T, Smiths Medical Instrument, China). Subsequently, Krebs-Ringer buffer containing 15, 30 and 60 μg mL$^{-1}$ morroniside was poured into the intestinal segment at a persistent flow rate of 0.2 mL min$^{-1}$ for about 1.5 h. Perfusion fluid samples gained from the outlet of intestinal segments were gathered every 15 min after equilibration for 30 min.

During the whole experiment, the exposed intestinal segments were maintained moist after cannulation with isotonic 0.9 % NaCl solution-moistened gauze and were kept at 37 °C. After *in situ* permeability studies, the radius and length of the duodenum, jejunum, ileum and colon segments were gauged (17).

$C_{\text{out(corr)}}$ (μg mL$^{-1}$, corrected outlet concentration of morroniside) and $P_{\text{eff}}$ (cm s$^{-1}$, quantitative measure of the rate of morroniside permeability across the biomembrane) were computed using the following equations:

$$C_{\text{out(corr)}} = \frac{C_{\text{out}} PR_{\text{in}}}{PR_{\text{out}}} \quad (2)$$

$$P_{\text{eff}} = \frac{Q \ln(C_{\text{in}} / C_{\text{out(corr)}})}{2\pi rL} \quad (3)$$

where $C_{\text{out}}$ (μg mL$^{-1}$) is the uncorrected outlet concentration of morroniside, $PR_{\text{in}}$ and $PR_{\text{out}}$ (mg mL$^{-1}$) are the inlet and outlet concentrations of phenol red, resp., $Q$ (0.2 mL min$^{-1}$) is the flow rate of the perfusate entering the rats' isolated intestinal segments, $C_{\text{out(corr)}}$ and $C_{\text{in}}$ are the corrected outlet and inlet concentrations of morroniside, resp., $r$ (cm) and $L$ (cm) are the radius and length of the perfused intestinal segment.

## *Cell culture*

Caco-2 cells (American Type Culture Collection, USA) between passage 40 and 50 were used and cultured at 37 °C in 5 % $CO_2$ at 90 % humidity in DMEM (high glucose)

medium containing 15 % fetal bovine serum, 1 % non-essential amino acid solution, 100 U $mL^{-1}$ penicillin and 1 % streptomycin. The DMEM medium was replaced twice a week. The cells were seeded in collagen-coated transwell inserts (0.4-μm pore size, 24-mm diameter; Millipore, USA) at a density of $3 \times 10^5$ cells per insert. The cells were cultured for 21 days after seeding. The DMEM medium was replaced every two days.

*Cytotoxicity test*

The cytotoxicity test of morroniside to Caco-2 cells was performed using the MTT assay. The Caco-2 cells in the logarithmic growth phase were seeded onto a 96-well plate at a density of $1 \times 10^5$ cells per well in 100 μL DMEM culture medium. After culturing at 37 °C in 5 % $CO_2$ for 3 days, the culture medium was replaced with 100 μL of morroniside solution in HBSS (pH 7.4) at concentrations of 6.4, 32, 160, 800 and 4000 μmol $L^{-1}$ and the HBSS (pH 7.4) was used as a blank control. Two hundred (200) μL of 5 mg $mL^{-1}$ MTT solution in HBSS was added to each well after the plate was incubated at 37 °C for 4 h, and then the plate was incubated for another 4 h. The medium was then replaced with 100 μL of DMSO. The absorbance of the mixture in the 96-well plate was measured at 570 nm. The cytotoxicity of morroniside at different concentrations was calculated by the percentage of absorbance relative to that of the blank control.

*Transport studies*

Only cultures with transepithelial electrical resistance (TEER) (Millicell ERS®, Millipore) values over 400 Ω $cm^{-2}$ were employed for the flux experiment. Before the experiments, the Caco-2 cell monolayers were washed twice with incubation medium (HBSS, pH 7.4, 137 mmol $L^{-1}$ NaCl, 5.36 mmol $L^{-1}$ KCl, 0.952 mmol $L^{-1}$ $CaCl_2$, 0.812 mmol $L^{-1}$ $MgSO_4$, 0.440 mmol $L^{-1}$ $KH_2PO_4$, 0.385 mmol $L^{-1}$ $Na_2HPO_4$, 25 mmol $L^{-1}$ D-glucose and 25 mmol $L^{-1}$ Hepes). Volumes of drug solution added to the apical (AP) and basolateral (BL) sides of the monolayer were 0.4 mL and 1.2 mL, resp. The permeability of morroniside (16, 80 and 400 μmol $L^{-1}$) under all the above conditions for both directions, from the AP-to-BL side and from the BL-to-AP side, was measured at 37 °C. A 50-μL aliquot of this incubation solution was withdrawn from the receiver compartment at 0, 15, 60 and 120 min.

$P_{app}$ and $P_{ratio}$ were calculated by the following equations:

$$P_{app} = \frac{\Delta Q}{\Delta t\, A\, C_0} \tag{4}$$

$$P_{ratio} = \frac{P_{app,(BL \rightarrow AP)}(\text{mean})}{P_{app,(AP \rightarrow BL)}(\text{mean})} \tag{5}$$

where $\Delta Q/\Delta t$ (μmol $s^{-1}$) is the permeation rate of morroniside, $A$ ($cm^2$) is the surface area of the epithelium (0.33 $cm^2$) and $C_0$ (μmol $L^{-1}$) is the initial donor concentration of morroniside on the loading side. The $P_{ratio}$ of morroniside was obtained by dividing the BL→AP permeability by the AP→BL permeability.

*Determination of morroniside by HPLC-MS/MS*

The LC-MS/MS method has been already validated for the determination of morroniside in rat plasma over a concentration range of 2.0–5000 ng $mL^{-1}$ with a lower limit of

quantification (*LLOQ*) of 2.0 ng mL$^{-1}$ (13). Chromatographic separation was carried out on an Inertsil C8-3 column using acetonitrile and water containing 1 mmol L$^{-1}$ sodium formate at 0.4 mL min$^{-1}$ for 6 min. Linear gradient elution was applied. No interferences from endogenous substances were found at the retention times of either morroniside or IS. The intra- and inter-day accuracy for morroniside ranged from 91.4 to 98.2 % and 93.4 to 102.0 %, resp. Intra- and inter-day RSD for morroniside ranged from 4.3 to 5.1 % and 5.0 to 7.7 %.

Data acquisition was performed using an API 5000 triple-quadrupole mass spectrometer coupled with an ESI source (Applied Biosystems/MDS SCIEX, USA) and Analyst software (version 1.5.1). The ESI source was operated with ion spray voltage of 5000 V and heater temperature of 550 °C. Gas settings were as follows: curtain gas 15 L h$^{-1}$, collision gas 5 L h$^{-1}$, ion source gas 1: 30 L h$^{-1}$, and ion source gas 2: 40 L h$^{-1}$. Quantification was carried out by multiple reaction monitoring (MRM) in a positive ionization mode. Hyperoside was used as an internal standard (IS). Transitions selected for quantification were *m/z* 429→267 for morroniside and *m/z* 487→324 for IS. Collision energy values for morroniside and IS were 35 and 40 V, respectively.

### *Biosample preparation*

Frozen biosamples were thawed at room temperature and vortex-mixed. Fifty μL of biosample was mixed with 50 μL water, 100 μL IS working solution (500 ng mL$^{-1}$) and 150 μL methanol and vortex-mixed for 1 min. After centrifugation at 14000 rpm at 4 °C for 10 min, 10 μL of supernatant was injected onto the LC-MS/MS system for analysis (13).

### *Statistical analysis*

Experimental data were expressed as mean ± standard deviation (SD). Statistical analysis was performed with the SPSS 20.0 software package (SPSS Inc., Chicago, IL, USA). Differences between the two groups were calculated by Student's *t* test. Significance of the differences between groups for continuous variables was evaluated with one-way analysis of variance (ANOVA), the mA value of $p < 0.05$ indicating statistical significance.

## RESULTS AND DISCUSSION

### In vivo *permeability of morroniside*

The LC-MS/MS method was successfully applied to the pharmacokinetic studies of morroniside at doses of 10 (*i.v.* and *p.o.*), 30 (*p.o.*), and 90 (*p.o.*) mg kg$^{-1}$ in rats. The pharmacokinetics of morroniside in rats within the used dosage ranges (10, 30 and 90 mg kg$^{-1}$) after oral administration was in accord with linear pharmacokinetic characteristics. The linear regression equation between $AUC_{(0-t)}$ and the dose was $y = 58.98x - 249.02$ ($R^2 = 0.9827$), while it was $y = 19.77x + 87.65$ ($R^2 = 0.9958$) between $C_{max}$ and the dose. Moreover, no apparent differences for *MRT*, $T_{1/2z}$, $T_{max}$, $V_d$ and $CL_z/F$ were observed regardless of increasing doses.

The $AUC_{(0-t)}$ of morroniside after an intravenous dose (10 mg kg$^{-1}$) to SD rats was 14673.84 ± 3512.36 ng h mL$^{-1}$, compared with 610.84 ± 361.61, 1160.42 ± 412.12 and 5149.45 ± 3557.32 ng h mL$^{-1}$ after an oral dose of 10, 30, 90 mg kg$^{-1}$ of morroniside, resp. The pharmacokinetic study of morroniside within the used dosage ranges exhibited linear dose-proportional

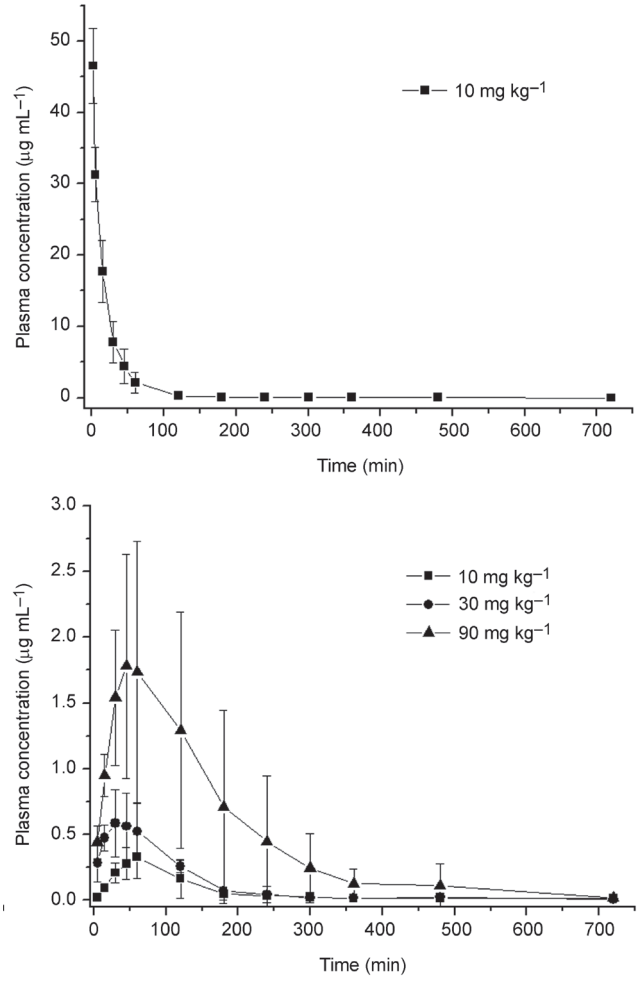


Fig. 1. Plasma concentration-time profiles of morroniside following: a) a single intravenous injection, and b) a single oral administration in rats (SD bars, $n = 5$).

pharmacokinetic characteristics and low bioavailability (4.3 %) in SD rats, which meant that only 4.3 % of the prototype drug was absorbed into blood circulation. The plasma concentration-time profiles of morroniside after intravenous and oral administration of morroniside to rats are shown in Figs. 1a,b with the corresponding pharmacokinetic parameters given in Table I.

## In situ *permeability of morroniside*

This was the first study to investigate the absorption mechanism of morroniside using a single-pass intestinal perfusion *in situ* model in SD rats. The stability of morroniside in

*Table I. Pharmacokinetic parameters of morroniside in rats*

| Parameter | Treatment (mg kg$^{-1}$)[a] | | | |
|---|---|---|---|---|
| | 10 (*i.v.*) | 10 (*p.o.*) | 30 (*p.o.*) | 90 (*p.o.*) |
| $AUC_{(0\text{-}t)}$ (ng h mL$^{-1}$) | 14673.84 ± 3512.36 | 610.84 ± 361.61 | 1160.42 ± 412.12 | 5149.45 ± 3557.32 |
| $AUC_{(0\text{-}\infty)}$ (ng h mL$^{-1}$) | 14688.29 ± 3509.75 | 628.86 ± 356.95 | 1191.68 ± 465.35 | 5215.45 ± 3610.10 |
| $MRT_{(0\text{-}t)}$ (h) | 0.38 ± 0.08 | 1.62 ± 0.49 | 1.43 ± 0.27 | 1.90 ± 0.58 |
| $T_{1/2z}$ (h) | 1.77 ± 0.35 | 1.26 ± 0.65 | 1.40 ± 1.17 | 1.24 ± 0.37 |
| $T_{max}$ (h) | 0.03 ± 0.00 | 1.00 ± 0.00 | 0.57 ± 0.34 | 1.05 ± 0.57 |
| $CL_z$ (L h$^{-1}$ kg$^{-1}$) | 0.71 ± 0.17 | 19.82 ± 9.02 | 27.59 ± 7.89 | 27.23 ± 19.45 |
| $V_z$ (L kg$^{-1}$) | 1.88 ± 0.76 | 36.86 ± 31.27 | 49.61 ± 35.04 | 47.95 ± 39.07 |
| $C_{max}$ (ng mL$^{-1}$) | 46530.00 ± 5238.63 | 329.60 ± 168.27 | 621.80 ± 227.07 | 1881.80 ± 881.34 |
| $F_{(0\text{-}\infty)}$ (%) | – | 4.3 | – | – |

[a] Mean ± SD, $n$ = 5.

*Table II. The* $P_{eff}$ *values of three concentrations of morroniside in different intestinal segments*

| Morroniside (μg mL$^{-1}$) | $P_{eff}$ value × 10$^6$ (cm s$^{-1}$)[a] | | | |
|---|---|---|---|---|
| | Duodenum | Jejunum | Ileum | Colon |
| 15 | 3.11 ± 1.71 | 3.63 ± 1.48 | 3.31 ± 1.98 | 3.09 ± 2.03 |
| 30 | 4.30 ± 2.15 | 3.96 ± 1.23 | 4.53 ± 0.94 | 4.31 ± 1.80 |
| 60 | 4.46 ± 1.52 | 4.13 ± 1.78 | 4.42 ± 1.73 | 4.28 ± 1.64 |

[a] Mean ± SD, $n$ = 4.

Krebs-Ringer buffer at 37 °C was investigated for at least 24 h, which showed that morroniside was stable under the above conditions (data not published). Its average effective permeability coefficient ($P_{eff}$ value) for transport across the small intestinal segments changed from (3.09 ± 2.03) × 10$^{-6}$ to (4.53 ± 0.94) × 10$^{-6}$ cm s$^{-1}$. The $P_{eff}$ values were basically consistent irrespective of increasing concentrations of morroniside at the measured concentrations from 15 to 60 μg mL$^{-1}$ after perfusion *via* duodenum, jejunum, ileum and colon segments (Table II). The $P_{eff}$ values were all close to 3.0 × 10$^{-6}$, which meant poor intestinal absorption (18) at three concentrations (15, 30, and 60 μg mL$^{-1}$).

## *Bi-directional transport of morroniside across Caco-2 cells*

The *in vitro* study investigated the bi-directional transport of morroniside across Caco-2. The MTT assay was used to investigate morroniside cytotoxicity to Caco-2 cells in order to establish appropriate practical concentrations for bi-directional transport research. The result indicated that morroniside showed no toxic effect to Caco-2 cells in the

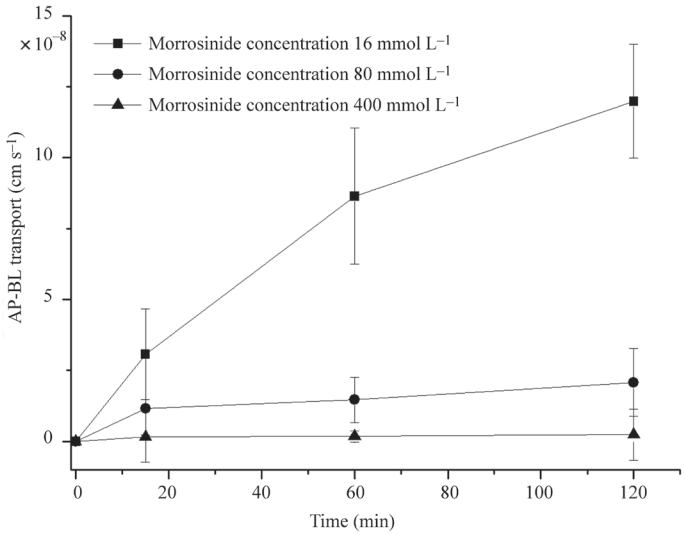


Fig. 2. Impact of time and concentration on morroniside absorption (SD bars, $n = 3$).

*Table III. The* $P_{ratio}$ *of morroniside*

| *t* (min) | $P_{ratio}$ at morroniside concentration[a] | | |
|---|---|---|---|
| | 16 (μmol $L^{-1}$) | 80 (μmol $L^{-1}$) | 400 (μmol $L^{-1}$) |
| 15 | 0.01 | 0.01 | 0.6 |
| 60 | 0.04 | 0.3 | 1.2 |
| 120 | 0.05 | 0.5 | 1.2 |

[a] Mean value, $n = 3$.

concentration range from 6.4 μmol $L^{-1}$ to 4.0 mmol $L^{-1}$. Morroniside concentrations (16, 80, and 400 μmol $L^{-1}$) were used to investigate the morroniside transport mechanism across the Caco-2 cell monolayer, which showed that the apparent permeability coefficient ($P_{app}$) values of morroniside ranged from (1.61 ± 0.53) × $10^{-9}$ to (1.19 ± 0.22) × $10^{-7}$ cm $s^{-1}$ for AP to BL. The efflux ratios ($P_{ratio}$) at three concentrations were all lower than 1.2.

It could be inferred that the active uptake transport was involved in the absorption process of morroniside, because the $P_{ratio}$ values decreased with the increase in morroniside concentration. In addition, according to the obtained $P_{ratio}$ values from all concentrations and time points ($P_{ratio}$ value < 1.5), it could be concluded that morroniside might not be a substrate for P-gp (19), but active uptake transports might be involved in the transport of morroniside across the Caco-2 cell monolayer. The impact of time on morroniside absorption at the above concentrations is presented in Fig. 2. The $P_{ratio}$ values of morroniside are summarized in Table III.

## CONCLUSIONS

In conclusion, the present research studied *in vivo*, *in situ*, and *in vitro* absorption of oral morroniside. The pharmacokinetics of morroniside in rats within the used dosage ranges exhibited linear dose-proportional pharmacokinetic characteristics and low bioavailability. The *in situ* and *in vitro* results indicated that the intestinal transport of morroniside obeyed a concentration-independent active transport mechanism and the P-gp was not involved in morroniside transport. This research provided a rational explanation for the poor absorption of morroniside observed in pharmacokinetic experiments. For the above reasons, further investigations would be required to improve morroniside bioavailability.

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*Acronyms, abbreviations, symbols.* – $A$ – surface area of the epithelium, AP – apical side, *AUC* – area under curve, BL – basolateral side, $C_0$ – initial donor concentration on the loading side, $CL_z$ – clearance, $C_{max}$ – peak concentration, $C_{out}$ – uncorrected outlet concentration, $C_{in}$ – corrected inlet concentration, $C_{out(corr)}$ – corrected outlet concentration, $\Delta Q/\Delta t$ – permeation rate, DMEM – Dulbecco's modified Eagle medium, $F$ – bioavailability, HBSS – Hank's balanced salt solution, $L$ – length of the perfused intestinal segment, MRM – multiple reaction monitoring, MRT – mean residence time, $P_{app}$ – apparent permeability coefficient values, MTT – 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, $P_{eff}$ – rate of permeability across a membrane, P-gp – P-glycoprotein, $PR_{in}$ – inlet concentration of phenol red, $PR_{out}$ – outlet concentration of phenol red, $Q$ – perfusion flow rate through the intestinal segment, $r$ – radius of the perfused intestinal segment, SD rats – Sprague Dawley rats, $T_{1/2z}$ – half-life, TEER – transepithelial electrical resistance, $T_{max}$ – peak time, $V_z$ – apparent volume of distribution.

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