Metabolism and plasma pharmacokinetics of isoorientin, a natural active ingredient, in Sprague-Dawley male rats after oral and intravenous administration

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Abstract
1. Several pharmacological effects have been revealed on isoorientin, suggesting its potential medicinal prospects. The metabolic and plasma pharmacokinetic profiles of isoorientin were investigated in rats.
2. For intra-gastric gavage, parent drug and three metabolites were detected in urine and feces by HPLC–MS/MS, but only one metabolite was found in plasma and identified as isoorientin 3'-or 4'-O-sulfate (M1) according to MS and UV absorbance spectra.
3. After a single i.v. administration of isoorientin (5, 10, or 15 mg/kg B.W.) in rats, linear pharmacokinetic property was observed with favorable terminal half-lives (1.67 ± 1.32–2.07 ± 0.50 h). After a single p.o. administration of isoorientin (150 mg/kg B.W.) in rats, plasma isoorientin concentration was low, but the concentration of M1 was comparatively high. Low systemic exposure of oral isoorientin in rats could result from its low aqueous solubility and extensive first-pass metabolism, and plasma concentration of M1 can be used as a biomarker of isoorientin intake. Isoorientin showed low oral bioavailability (8.98 ± 1.07%), and had about 6% or 45% dose recovery in urine or feces, respectively, 72 h after intra-gastric gavage.
4. These studies are the first to describe the pharmacokinetics of isoorientin via i.v. or p.o. dosing, providing important information for understanding its process in vivo.

Keywords
Isoorientin, metabolism, pharmacokinetics

Introduction
Isoorientin (6-C-glucopyranosyl-3',4',5,7-tetrahydroxylavone, Figure 1) is a natural bioactive flavonoid C-glycoside, frequently occurring in some edible or medicinal plants, e.g. Cymbopogon citratus (DC.) Stapf (Francisco et al., 2014), Gentianopsis barbata var. sinensis Ma (Cui et al., 2013), yellow passion fruit (López-Vargas et al., 2013), Aloe section Pictae (Grace et al., 2010), Neurada procumbens L. (Marzouk et al., 2014), and Dryoathyrium boryanum (Wild.) Ching (Cao et al., 2013). Recently, a variety of pharmacological effects have been revealed on isoorientin, including antioxidant (Yao et al., 2012; Yuan et al., 2013a), anti-inflammation (Yuan et al., 2014), anti-cancer (Pacifico et al., 2010; Yuan et al., 2012, 2013b) and anti-diabetic effects (Alonso-Castro et al., 2012; Li et al., 2009).

With the increasing knowledge of a potential beneficial role of isoorientin in human health, isoorientin gradually showed potential medicinal prospects and deserves to be further investigated. However, till date, reports on isoorientin only focused on the investigations of extraction, isolation and determination methods or pharmacological effects. There is little information referring to ascertain metabolites, pharmacokinetics or bioavailability of isoorientin. Sheng et al. (2014) simultaneously determined three secoiridoid glycosides and three flavonoid glycosides (including isoorientin) in rat plasma, bile, urine and feces after single-oral administration of the Swertia pseudochinensis extract (10 g/kg B.W.) by LC-MS/MS. The plasma pharmacokinetics and excretion profiles of the multiple components were evaluated. However, there was a probability that the pharmacokinetics profiles of isoorientin obtained in the above-mentioned study was influenced by the possible pharmacokinetic interactions among the multiple components. The pharmacokinetics of individual isoorientin by oral or intravenous administration was not reported, and the metabolism and bioavailability profiles of isoorientin remain unknown. Therefore, the present study aimed to identify the major metabolites of isoorientin for disclosing the metabolism pathways, and evaluate pharmacokinetics and bioavailability of isoorientin in rats. The results will undoubtedly contribute to a better understanding of the disposition and pharmacokinetics of...
isoorientin, and provide an important information on ascertaining its process in vivo for further non-clinical or clinical investigations.

Materials and methods

Chemicals

Standard compound isoorientin (99% purity by HPLC) was purchased from Shanghai Ronghe Medicine Technology Development Co. Ltd. (Shanghai, China). The internal standard (IS) puerarin was purchased from Guangdong Shunfeng Pharmaceutical Co. Ltd. (Foshan, China). The chemical structures of isoorientin and puerarin are shown in Figure 1. HPLC grade methanol was purchased from Merck (Darmstadt, Germany) while acetic acid glacial (HPLC-grade) was from Shanghai Jingchun Reagent Co. Ltd. (Shanghai, China). Double-distilled water was used for all the preparations. Physiologic saline was obtained from Fuzhou Haiwang Fuyao Pharmaceutical Co. Ltd. (Fuzhou, China). Tween 80 and L-ascorbic acid were purchased from Sinopharm chemical regent Co. Ltd (Shanghai, China).

Animals

Male Sprague-Dawley rats (6–7 weeks, 250 ± 50 g) were obtained from Shanghai Slac Laboratory Animal Co. Ltd (Shanghai, China). They were individually housed in cages in an air-conditioned room at temperature of 25 ± 2°C and a relative humidity of 50 ± 10% with a 12-h light/dark cycle, and allowed food and water spontaneously. All animals were handled according to an approved animal use protocol of Fujian Medical University. Animals were given free access to water but were fasted for 12 h before an experiment and for 2 h after drug administration.

Study design

For intravenous administration, isoorientin was suspended with 0.5% tween 80–99.5% physiologic saline to prepare 4 mg/mL dosing solution. For intra-gastric administration, isoorientin was suspended with 2% tween 80–98% physiologic saline to prepare 20 mg/ml dosing solution. Isoorientin was administered intravenously via the tail vein and by oral gavage to four parallel groups (5, 10, and 15 mg/kg for intravenous administration, n = 3 for each group and the dosing solutions were diluted unified into 1 mL with physiologic saline; 150 mg/kg for oral administration, n = 4 and the dosing solutions were unified diluted into 2 mL with physiologic saline) for the pharmacokinetic study, and by oral administration (150 mg/kg, n = 3) for the metabolite profiling study. For plasma pharmacokinetic study, approximately 250 μL of blood was collected from the cut-tail before dosing and at the following times after dosing: 0.0167, 0.25, 0.5, 0.75, 1, 2, 4, 6, 10, 24, 32, 48, and 72 h. The injection site was selected at about the 1/2 station of the rat tail. After injection, the rat tail was cleaned carefully with alcohol cotton balls around the injection site to remove the drug residual as much as possible. Plasma was obtained by centrifugation at 4°C and stored at −20°C until analysis. For metabolite study, the plasma samples used were the same ones for pharmacokinetic study. The urine and feces samples were collected at the time intervals: 0–2, 2–4, 4–6, 6–8, 8–24, 24–32, and 32–72 h in plastic tubes containing 200 μL of ascorbic acid (0.1%, w/v) and the collected samples were temporarily placed over

Figure 1. Chemical structures of isoorientin (M0), its major metabolite M1 (isoorientin 3'- or 4'-O-sulfate), M2 (3'-O-methylated isoorientin), M3 (4'-O-methylated isoorientin), and puerarin.
wet ice. Subsequently, the collected urine and feces samples were stored at \(-20^\circ\)C until analysis.

**LC-MS/MS and HPLC-DAD procedures for metabolites analysis**

A Shimadzu Liquid Chromatograph Mass Spectrometer 8040 (Shimadzu, Japan) equipped with an electrospray ionization (ESI) source system and a Shimadzu LC20A HPLC system (Shimadzu, Japan) consisting of a vacuum degasser, a binary pump, a column oven and an autosampler were used to identify the metabolites of isoorientin in rat plasma, urine or feces samples. The MS conditions were as follows: ion mode, positive/negative; block heating temperature, 400°C; desolvation line temperature, 250°C; dry gas (nitrogen), 12 mL/min; and auxiliary gas (nitrogen), 3 mL/min. The full-scan mass spectrum was collected in the mass range from \(m/z\) 100 to 1000. Furthermore, MS/MS spectra were obtained for selected precursor ions through collision-induced dissociation with neutral gas (argon) molecules in the collision cell, which could provide characteristic fragment ions of each metabolite. The relative collision energy was set at 35%. In order to obtain the ultraviolet spectra of parent drug and metabolites, an HPLC system (Agilent 1260, Santa Clara, CA) equipped with a quaternary pump, an autosampler, a column oven and a diode array detector was used.

To simultaneously detect the metabolites, the column (Ultimate XB-C18, i.d. 100 mm \(\times\) 4.6 mm, with 3.5-\(\mu\)m particle size) with a Ultimate Guard column XB-C18 (5 \(\mu\)m) was eluted with a binary mixture of (A) 0.5% HAc-H2O and (B) acetonitrile at a total flow rate of 0.5 mL/min. The gradient program is shown as follows: initial 17% (A), at 0–10 min, linear change from 17% to 22% (B), at 10–11 min, linear change from 22% to 95% (B), at 11–14 min, at 95% (B), at 14–15 min, linear change from 95% to 17% (B). Re-equilibration duration was 10 min between individual runs. The column temperature was set at 30°C and elution was monitored at 200–400 nm on the diode array detector.

For metabolites identification, 100 \(\mu\)L urine or plasma sample was mixed with 300 \(\mu\)L methanol. Then, it was shaken for 2 min and centrifuged at 16,000 rpm at 4°C for 15 min. A total of 250 \(\mu\)L of the supernatant was transferred to a test tube, followed by evaporation by nitrogen stream, and the residue dissolved in a 100-\(\mu\)L 80% methanol-water (v/v) was shaken for 2 min. The re-dissolved was centrifuged at 16,000 rpm at 4°C for 15 min. A total of 5 \(\mu\)L of the supernatant was injected into HPLC-DAD for quantification analysis (methodology validation data were provided in the “Supplementary material”).

For oral administration, the plasma (100 \(\mu\)L) was treated with the same treatment procedures to those for the intravenous administration, except that the added IS was 20 \(\mu\)g/mL puerarin in methanol, the serial concentration of isoorientin solutions was diluted 10-fold with methanol, and the analysis was performed by triple quadrupole LC-MS/MS with multiple reaction monitoring mode (ESI in the negative ion mode using MRM of transitions of \(m/z\) 447 \(\rightarrow\) 327 for isoorientin and \(m/z\) 415 \(\rightarrow\) 267 for puerarin) (methodology data were not provided). Meanwhile, urine sample was also treated with the same treatment procedures to those for the intravenous administration and determined by HPLC-DAD. Since it was difficult to obtain the standard references of metabolites, the calibration curves of isoorientin were adopted to estimate the contents of metabolites (called as “semi-quantitative” in this study) in rat biosamples. All the semi-quantitative of metabolites in plasma and urine for oral administration were performed by HPLC-DAD with the linearity equations of isoorientin and with the peak area ratio of metabolite to IS as the independent variable \(x\). For feces analysis, all samples from each rat in 72 h were mixed and soaked in 5-fold amount (g/mL) of 80% methanol-water (v/v) for 30 min. Then, it was shaken for 10 min, followed by filtration with micro-pore filtration membrane (pore size 0.22-\(\mu\)m; Varian Inc., Palo Alto, CA). A total of 200 \(\mu\)L of the filtrate was centrifuged at 16,000 rpm at 4°C for 15 min. A total of 5 \(\mu\)L of the supernatant was injected for HPLC-DAD for quantification analysis.

For oral administration, the plasma (100 \(\mu\)L) including study samples or control plasma (for standard and quality controls) was mixed with 10 \(\mu\)L of IS (200 \(\mu\)g/mL puerarin in methanol). Subsequently, 10 \(\mu\)L of standard assay solutions (a serial concentration of isoorientin solutions) were added into the standard or quality control samples. Then, 300 \(\mu\)L of methanol was added into the mixture, followed by shaking for 2 min at room temperature and followed by centrifugation for 15 min at 16,000 rpm at 4°C. A total of 250 \(\mu\)L of the supernatant was transferred to a test tube, followed by evaporation by nitrogen stream, and the residue dissolved in a 100-\(\mu\)L 80% methanol-water (v/v) was shaken for 2 min. The re-dissolved was centrifuged at 16,000 rpm at 4°C for 15 min. A total of 5 \(\mu\)L of the supernatant was injected into HPLC-DAD for quantification analysis.

**Pharmacokinetic analysis**

Pharmacokinetic parameters were calculated using a non-compartmental model with DAS 2.0 software (Chinese Pharmacologic Society, Beijing, China). The area under the plasma concentration versus the time curve-up to the last quantifiable time (AUC\(_{0\rightarrow t}\)) was calculated by the linear trapezoidal rule. The AUC up to infinity (AUC\(_{0\rightarrow\infty}\)) was calculated by the following formula:

\[
AUC_{0\rightarrow\infty} = AUC_{0\rightarrow t} + C_{last}/\lambda_z
\]

where \(C_{last}\) and \(\lambda_z\) are the plasma concentration at the last quantifiable time and the elimination rate constant relative to...
with the terminal phase, respectively. Oral bioavailability ($F_{oral}$) was calculated as follows: ($AUC_{0-\infty}$ for oral dose)/($AUC_{0-\infty}$ for intravenous dose), corrected for the difference in dose levels. For three i.v. doses, three $F_{oral}$ values could be obtained and the mean $F_{oral}$ was calculated by averaging the three $F_{oral}$ values obtained.

**Results**

**Identification of isoorientin metabolites in urine after oral administration of isoorientin in rats**

Urine samples were collected after single-oral administration of 150 mg/kg isoorientin in rats. Urine samples were analyzed by HPLC-DAD and LC-MS/MS, and isoorientin and three metabolites were detected in the chromatography system with a retention time of 7.34 min (M0), 8.28 min (M1), 11.88 min (M2), and 12.75 min (M3), respectively (Figure 2A).

The positive ion LC-MS spectrum of M0 showed a protonated molecule [M + H]$^+$ at $m/z$ 449. The LC-MS/MS spectrum of the ion [M + H]$^+$ at $m/z$ 449 showed major product ions at $m/z$ 299 and 329. Meanwhile, the negative ion LC-MS spectrum of M0 showed a deprotonated molecule [M – H]$^-$ at $m/z$ 447. The LC-MS/MS spectrum of the ion [M – H]$^-$ at $m/z$ 447 showed two major product ions at $m/z$ 299 and 327. The proposed fragmentation scheme for M0 is shown in Supplemental Figure 1. The UV spectrum of M0 was in accordance with the standard of isoorientin (Figure 2B) with three maximum absorption wavelengths, $\lambda_{max1} = 255$ nm, $\lambda_{max2} = 270$ nm and $\lambda_{max3} = 348$ nm. Furthermore, the LC-MS/MS spectrum and HPLC retention time of M0 were identical to those of the isoorientin (Figure 2A). Based on these results, M0 was identified as the parent drug, isoorientin.

The UV spectrum of metabolite M1 is similar to that of isoorientin (Figure 2B) with three maximum absorption wavelengths, $\lambda_{max1} = 255$ nm, $\lambda_{max2} = 270$ nm and $\lambda_{max3} = 334$ nm, suggesting that M1 contains the 6-C-Glucopyranosyl flavone carbon skeleton. The $\lambda_{max3}$ of M1, resulted from the absorption of the cinnamyl group, is 12 nm less than that of isoorientin, suggesting that the B cycle is substituted at the 3’- or 4’-O position. In MS analysis, M1 showed [M + H]$^+$ and [M – H]$^-$ ions at $m/z$ 529 and 527, which were 80 Da higher than those of isoorientin and confirming that M1 was the sulfonated isoorientin. For MS/MS analysis in positive ion mode (Figure 2C), the neutral losses of 134, 142, 146, 176, 200, 204, 218 and 230 Da from [M + H]$^+$ produced fragment ions at $m/z$ 395 ([M + H–SO$_3$–3*H$_2$O]+), 379 ([M + H–SO$_3$–2*H$_2$O]+), 365 ([2*X–SO$_3$–2*H$_2$O]+), 353 ([0,2*X–SO$_3$–2*H$_2$O], 329 ([0,2*X–SO$_3$–2*H$_2$O–CO]), 311 ([0,2*X–SO$_3$–H$_2$O], and 299 ([0,1*X–SO$_3$], respectively. In negative ion mode, the precursor ion [M – H]$^-$ generated [M – H–SO$_3$], [M – H–SO$_3$–H$_2$O], [0,3*X–SO$_3$], [0,3*X–SO$_3$–H$_2$O–CO], and 299 ([0,1*X–SO$_3$], respectively. These data further supported that sulfation sites of isoorientin for M1 could occur at 3’- or 4’-OH groups. The exact position of the sulfated moiety could not be identified at this stage. In addition, probably, it was two co-eluted isomeric sulfate conjugates, which was not separated by HPLC. According to these results, M1 was tentatively identified as isoorientin 3’- or 4’-O-sulfate and the proposed fragmentation scheme of M1 is shown in Figure 3.

The metabolite M2 and M3 had the same UV spectrum as isoorientin (Figure 2B) with three maximum absorption wavelengths, $\lambda_{max1} = 255$ nm, $\lambda_{max2} = 270$ nm and $\lambda_{max3} = 348$ nm, suggesting that M2 and M3 also contain the 6-C-Glucopyranosyl flavone carbon skeleton of isoorientin. Both M2 and M3 showed a protonated molecule [M + H]$^+$ at $m/z$ 463 and a deprotonated molecule [M – H]$^-$ at $m/z$ 461 for MS analysis in positive and negative ion modes, respectively. The LC-MS/MS spectrum of the ion [M + H]$^+$ at $m/z$ 463 showed major product ions at $m/z$ 313 and 343. Meanwhile, the LC-MS/MS spectrum of the ion [M – H]$^-$ at $m/z$ 461 also showed two major product ions at $m/z$ 298 and 341. All these data supported that both of M2 and M3 are methylated isoorientin at the site of 3’- or 4’-O. Since 3’-O methylation luteolin is eluted faster than 4’-O methylation luteolin (Chen et al., 2011), 3’-O methylation isoorientin should also be eluted faster than 4’-O methylation isoorientin. Therefore, M2 and M3 were tentatively identified as 3’-O-methylated isoorientin and 4’-O-methylated isoorientin, respectively. The proposed fragmentation scheme for M2 and M3 is shown in Supplemental Figure 2.

In addition, isoorientin in the urine samples (collected with metabolism cages at the time interval: 0–2, 2–4, 4–6, 6–8, 8–24, 24–32, and 32–72 h) were quantified by the developed HPLC-DAD method. Metabolites M1, M2 and M3 in the urine samples were semi-quantified by the developed HPLC-DAD method. As shown in Figure 4A, the renal excretion of all the four compounds reached maximum rates at 6–8 h. The percentage of renal excretion accumulation were 1.92%, 0.91%, 2.31%, and 1.34% for isoorientin, M1, M2, and M3, respectively, in 72 h after intra-gastric gavage of 150 mg/kg isoorientin (Figure 4B). Hence, the total isoorientin excreted in urine was about 6% of the dose. The sulfated metabolite M1 occupied about 30% of the excreted in urine and the total methylated metabolites were about 4% of the dose, which was approximately half of the excreted in urine.

**Metabolites in blood after oral administration of isoorientin in rats**

With the same analysis conditions with those for urine analysis, blood samples were analyzed by HPLC-DAD and LC-MS/MS, and only isoorientin and metabolite M1 were detected in circulating system (Supplemental Figure 3).

**Metabolites in feces after oral administration of isoorientin in rats**

Also, with the same analysis conditions with those for urine analysis, feces samples were analyzed by HPLC-DAD and LC-MS/MS, and isoorientin prototype, metabolites M1, M2, and M3 could be detected in feces samples (Supplemental Figure 4). The total amount of isoorientin, in feces samples in 72 h were quantified by the developed HPLC-DAD method. Meanwhile, metabolites M1, M2 and M3 in the samples were also semi-quantified by the developed HPLC-DAD method. The mean elimination amounts were 41.13%, 1.37%, 1.55%, and 0.96% for isoorientin, M1, M2, and M3, respectively, in 72 h after a single intra-gastric gavage of 150 mg/kg isoorientin. Hence, the total isoorientin excreted in feces was about 45% of the administration dose.
Figure 2. Identification of isoorientin and its metabolites in urine after oral administration of 150 mg/kg isoorientin in rats. A, HPLC chromatograms of blank rat urine (A1), blank urine spiked with isoorientin (A2), and 4–6 h urine (A3) after oral administration of 150 mg/kg isoorientin in rat. B, UV spectra of M0 (isoorientin), M1, M2, and M3 in urine by HPLC-DAD. C, MS/MS spectra of M0, M1, M2, and M3. mAU, milli-arbitrary units.
Pharmacokinetic results

After quantification of isoorientin, plasma isoorientin concentration–time curves were established (Figure 5A and B). Non-compartmental analyses were conducted and the pharmacokinetic parameters for intravenous administration and intra-gastric gavage of isoorientin in rats are summarized in Tables 1 and 2, respectively. For intravenous dose, the parameters $t_{1/2}$, Vdss, CLtot, and MRT almost remain unchanged with the dose increasing (Table 1), and especially, the AUC exhibited a dose-proportional increase in rats (5, 10, and 15 mg/kg) (Figure 5A and C). Based on these results, the pharmacokinetics of isoorientin was characterized as a linear kinetic process. Compared to the intravenous administration, the intra-gastric gavage showed low systemic plasma clearance (0.01 mL/h in the rat) and apparent volume of distribution (0.08 mL). The reason could be that the low-aqueous solubility of the drug causes the dissolution-limited absorption for oral administration. As a matter of course, the oral bioavailability of isoorientin was also low (The mean $F_{oral}$ calculated was 8.98% in rats). Nevertheless, the half-lives for both of intravenous administration and intra-gastric gavage in rats were favorable (1.67 ± 1.32 ~ 2.07 ± 0.50 h and 6.22 ± 2.24 h, respectively). Especially, from Figure 5(B), a double peak for isoorientin can be found, which suggested that it could experience an entero-hepatic cycle process for intra-gastric gavage. The $t_{1/2}$ was long and $T_{max}$ was 0.58 h for rats for oral dose. In addition, a large amount of the metabolite M1 could be detected for intra-gastric gavage to rats. Because of lacking M1 standard reference, the “semi-quantitative” of metabolite M1 in rat plasma was performed with the linearity equation of isoorientin. The plasma M1 concentration–time curve was predicted (Figure 5D). The mean $t_{1/2}$ of metabolite M1 (7.5 h) was similar to that of isoorientin (6.22 h) for single intra-gastric gavage of isoorientin.

Discussion

The objective of this study was to examine the metabolism and pharmacokinetics of isoorientin in rats for the preclinical evaluation of this drug candidate.

Glucuronidation was considered as a major metabolic pathway for flavonoids such as quercetin (Galijatovic et al.,...
2001), luteolin (Chen et al., 2011), and puerarin (Luo et al., 2012). Puerarin, a structurally similar flavonoid C-glycoside (daidzein-8-C-glucoside) to isoorientin, was mainly metabolized as puerarin-7-O-glucuronide in rats urine after intravenous administration of 50 mg/kg of puerarin, as previously shown (Luo et al., 2012). In vitro, a UDP-glucuronosyltransferase (UGT) reaction screening experiment also demonstrated that formation of puerarin-7-O-glucuronide was mainly catalyzed by UGT1A1 (Luo et al., 2012). However, to our surprise, there was no glucuronidation metabolite of isoorientin found in rat blood, urine or feces, regardless of intra-gastric gavage or intravenous administration of isoorientin. The result suggests that glucuronidation could not be a preferential metabolism pathway for isoorientin in rats.

Sulfation or methylation was also common metabolic pathways for flavonoids (Havsteen, 2002), such as quercetin (Galijatovic et al., 2001), apigenin (Gradolatto et al., 2004; Hu et al., 2003), catechin (Donovan et al., 1999), (-)-epicatechin (Vaidyanathan & Walle, 2002), and luteolin (Gradolatto et al., 2004). Indeed, in the present study, besides the prototype isoorientin, one sulfated metabolite (M1), and two methylated metabolites (M2 and M3) could be found in rat urine and feces after intra-gastric gavage of isoorientin (Figure 1). To the best of our knowledge, it is the first time to ascertain the sulfated and methylated metabolites of isoorientin, which were chromatographically separated and directly identified without enzyme hydrolysis. Because the sulfation and methylation of flavonoids were mostly catalyzed by sulfotransferase (SULT) and catechol O-methyltransferase (COMT), respectively, it is reasonable to speculate that both

Figure 5. Plasma isoorientin concentration–time curves for intravenous (A) and intra-gastric (B) administration (n = 3); the inserted is the plot (C) of dose versus AUC0-t for intra-gastric administration (n = 3); plasma isoorientin 3' or 4'-O-sulfate (M1) concentration–time curve (D) for intra-gastric administration of isoorientin (n = 4).

Table 1. Plasma pharmacokinetic parameters of isoorientin after intravenous administration in rats (mean ± SEM, n = 3).

| Parameters | Unit   | 5     | 10    | 15    |
|------------|--------|-------|-------|-------|
| t1/2 (h)   |        | 1.67 ± 1.32 | 2.07 ± 0.50 | 2.00 ± 0.89 |
| Vdss (mL)  |        | 333.91 ± 269.85 | 424.69 ± 111.65 | 452.83 ± 165.16 |
| CLtot (mL/h)|       | 130.36 ± 22.47 | 146.94 ± 53.79 | 163.22 ± 21.51 |
| AUC0→t (µg/mL × h)| | 9.08 ± 2.09 | 17.20 ± 5.43 | 22.17 ± 2.19 |
| AUC0→∞ (µg/mL × h)| | 9.75 ± 1.86 | 18.10 ± 5.55 | 23.23 ± 2.85 |
| MRT0→t (h) |        | 0.53 ± 0.11 | 0.81 ± 0.27 | 0.60 ± 0.22 |
| MRT0→∞ (h) |        | 0.99 ± 0.43 | 1.23 ± 0.34 | 0.94 ± 0.48 |

Table 2. Pharmacokinetic parameters of isoorientin after intra-gastric administration in rats (mean ± SEM, n = 4).

| Parameters | Unit   | 5     | 10    | 15    |
|------------|--------|-------|-------|-------|
| t1/2 (h)   |        | 6.22 ± 2.24 | 5.85 ± 0.14 | 10.29 ± 5.23 |
| Vdss (mL)  |        | 0.08 ± 0.06 | 0.01 ± 0.01 | 0.01 ± 0.01 |
| CLtot (mL/h)|       | 21.32 ± 5.23 | 23.61 ± 17.86 | 23.23 ± 2.85 |
| AUC0→t (µg/mL × h)| | 2.13 ± 5.43 | 22.17 ± 2.19 | 23.23 ± 2.85 |
| AUC0→∞ (µg/mL × h)| | 4.77 ± 1.51 | 7.22 ± 2.00 | 8.98 ± 1.07 |

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of the SULT and COMT were involved in the metabolism of isoorientin in rats.

As known, SULT is a sulfation metabolism enzyme system and generally classified into six subfamilies, SULT1, SULT2, SULT3, SULT4, SULT5, and SULT6 (Blanchard et al., 2004; Takahashi et al., 2009). Among them, SULT1 is extendedly expressed in many tissues and organs, such as liver, gastrointestinal tract and lung, mainly responsible for catalyzing the sulfation of phenolics in vivo. Reasonably, it was inferred that SULT1 might take part in the metabolism of isoorientin in rats. The proposed pathway for sulfation of isoorientin is shown in Figure 6. However, 3'-O-sulfated and 4'-O-sulfated isoorientins have similar partition coefficients, it was difficult to separate them by HPLC. Thus, currently, it lacks evidence to ascertain the exact sulfation site of M1 and further efforts are required.

COMT is a phase II enzyme responsible for O-methylation of catechols. Compounds containing the catechol moiety, such as dopamine, norepinephrine and luteolin, were also the substrate of COMT (Chen et al., 2011). In the present study, the formation of two methylation metabolites M2 and M3 demonstrated that isoorientin might also be a substrate of COMT in rats (Figure 6).

Noticeably, in rat plasma, the prototype isoorientin and sulfated isoorientin (M1) could be detected, and the methylated metabolites M2, and M3 could not be found by the developed HPLC-DAD and HPLC-MS methods after intra-gastric gavage of isoorientin. However, all the four substances (isoorientin, M1, M2, and M3) could be detected in rat urine and feces. It was inferred that M2 and M3 could be excreted rapidly via urinary system (Figure 4B), so that their concentrations in rat blood were below the limit detection of the methods developed after intra-gastric gavage of isoorientin. Interestingly, after intravenous administration of isoorientin (5, 10, or 15 mg/kg dose), a large amount of the prototype drug could be detected, and few of the three metabolites M1, M2 and M3 could be found in rat plasma. These results demonstrated that the hepatic or gastric first-pass effects could not be ignored for the oral absorption of isoorientin in rats, and liver could be a dominant metabolism organ for the sulfation of isoorientin via P.O. administration.

The metabolite M1 showed a high systematic exposure in rats and the long half-life (7.50 ± 0.74 h) of M1 is similar to that of isoorientin (6.22 ± 2.24 h) in rats. The estimated mean C_max of M1 (64.40 μg/mL) was four times higher than that of isoorientin (10.29 μg/mL), suggesting that the metabolite M1 was easy to be determined by HPLC method. Thus, for oral administration, plasma concentration of M1 could be used as a biomarker of isoorientin intake.

The total isoorientin eliminated from feces and urine was about 51% of the dose for intra-gastric gavage. It suggested that certain other metabolism pathways could effect the remaining 49% isoorientin. Putatively, isoorientin could be hydrolyzed in the gastrointestinal tract and degraded into small-molecule phenols and various aromatic acids, as in the metabolisms of quercetin (Walle et al., 2001) and dietary

Figure 6. Proposed metabolism pathway of isoorientin. Metabolites: M1 (isoorientin 3'- or 4'-O-sulfate), M2 (3'-O-methylated isoorientin), and M3 (4'-O-methylated isoorientin).
procyanidins (Gonthier et al., 2003), flavone C-glucosides (Zhang et al., 2007). Further efforts were required for revealing other excretion pathways and mechanisms to understand its in vivo process and thereby contributing further in non-clinical or clinical investigations.

Conclusions

In summary, the use of HPLC-ESI/MS in multi-stage full-scan mode allowed us to identify the metabolites of isoorientin in rat plasma, urine, and feces samples. For intra-gastric gavage, parent drug isoorientin and three metabolites M1, M2, and M3 were identified in rat urine and feces. Both of the two enzymes SULT1 and COMT could be involved in the metabolism of isoorientin in rats. The hepatic or gastric first-pass effects and low-aqueous solubility of isoorientin could be the major factors for limiting the oral absorption of isoorientin in rats, leading to a low-oral bioavailability of isoorientin in rats. Due to the linear pharmacokinetics and a favorable half-life of isoorientin, it was possible to maintain an effective concentration of isoorientin in vivo in a moderate long period of time (such as, beyond 1 h) for fulfilling the aim of treating diseases with the intravenous administration mode.

Declaration of interest

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Supplementary material available online
Supplementary Figure S1–S4