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RESEARCH ARTICLE

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Studies on oral bioavailability and first-pass metabolism of withaferin A in rats using LC–MS/MS and Q-TRAP

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Abstract

Withaferin A (WA) is one of the major bioactive steroidal lactones with extensive pharmacological activities present in the plant *Withania somnifera*. The absolute oral bioavailability of WA remains unknown and human-related *in vitro* data are not available. Therefore, in the present study, the absolute oral bioavailability of WA in male rats and the *in vitro* screening of absorption factors by Q-trap and LC–MS/MS analysis were conducted to explore possible clinical properties of WA. The developed and validated analytical methods were successfully applied to the pharmacokinetic studies and *in vitro* measurement of WA. The oral bioavailability was determined to be 32.4 ± 4.8% based on intravenous (5 mg/kg) and oral (10 mg/kg) administrations of WA in male rats. The *in vitro* results showed that WA could be easily transported across Caco-2 cells and WA did not show as a substrate for P-glycoprotein. Moreover, the stability of WA was similar between male rat and human in simulated gastric fluid (stable), in intestinal microflora solution (slow decrease) and in liver microsomes (rapid depletion, with a half-life of 5.6 min). As such, the first-pass metabolism of WA was further verified by rat intestine-liver *in situ* perfusion, revealing that WA rapidly decreased and 27.1% remained within 1 h, while the content of three major metabolites (M1, M4, M5) identified by Q-trap increased. This perfusion result is consistent with the oral bioavailability results *in vivo*. The first-pass metabolism of WA might be the main barrier in achieving good oral bioavailability in male rats and it is predicted to be similar in humans. This study may hold clinical significance.

KEYWORDS

bioavailability, first-pass metabolism, *in situ* intestine-liver perfusion, metabolite identification, withaferin A

1 | INTRODUCTION

Withania somnifera (or Ashwagandha) is a plant widely used as an Indian traditional herbal medicine because it displays several pharmacological activities such as antitumor, antioxidant, anti-inflammatory and immunomodulator, as well as for treatment of disorders of the central nervous system (Dar, Hamid, & Ahmad, 2015; Dhami, Chang, & Gambhir, 2017; Mir, Khazir, Mir, Hasan, & Koul, 2012; Patil et al., 2013). Withaferin A (WA) is one of the major bioactive steroidal lactones in *W. somnifera* extract (Dar et al., 2015). Recently, it has been reported that WA possesses potent antiglioma activity in an orthotopic glioma mice model, and its pharmacological activity on Alzheimer's disease has received attentions, in addition to its anti-

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diabetic and powerful weight-loss effect (Choudhary et al., 2005; Lee et al., 2016; Mcfarland et al., 2013; Santagata et al., 2012).

Although *W. somnifera* extracts for oral administration have been available on the market for many years, only mice have been used to study the nonsystemic pharmacokinetics of WA. Thus, the absolute oral bioavailability of WA remains unknown and human-related *in vitro* data are not available (Gambhir et al., 2015; Patil et al., 2013; Thaiparambil et al., 2011). If the oral bioavailability of WA is calculated by combining the results in mice from two different studies—oral administration of 10 mg/kg WA and intravenous administration of 5 mg/kg WA—the oral bioavailability will be ~9%, which might not be satisfactory for its application in oral administration (Ku, Han, & Bae, 2014; Patil et al., 2013). The authentic oral bioavailability of WA and the factors influencing it should be assessed.

A previous report showed that the main factors affecting oral bioavailability are intestinal permeability, metabolism and anatomical–physiological factors (gastrointestinal pH, bacterial microflora, dimensions and surface areas of the gastrointestinal tract; El-Kattan, Hurst, Brodfuehrer, & Loi, 2011). As regards some high-permeability drugs, low oral bioavailability and poor therapeutic efficacy may be due to the first-pass effect (Svensson, Sandström, Carlborg, Lennernäs, & Ashton, 1999). Therefore, in the present study, the oral bioavailability of WA was recalculated in rats, whose intestinal permeability is similar to that in humans with correlation $r^2 = 0.8$ based on the results from *in situ* intestinal perfusion in rats and *in vivo* human jejunal perfusion for 14 drugs (Cao et al., 2006). The main factors affecting oral bioavailability were also explored based on the *in vitro* screenings of absorption-related factors by Q-trap MRM–IDA–EPI (multiple reaction monitoring–information dependent acquisition–enhanced product ion) and LC–MS/MS analysis. The *in vitro* studies using human cells and samples and further understanding of the absorption, metabolism and transport of WA may come with clinical significance.

2 | EXPERIMENTAL

2.1 | Chemicals, reagents and animals

Withaferin A (purity >99.0%) and Alisol C 23-acetate (internal standard, IS, purity >99.0%) were purchased from China Chengdu Push Bio-technology Co. Ltd. Digoxin and ketoconazole were purchased from the National Institutes for Food and Drug Control (Beijing, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, 0.25% trypsin–EDTA, penicillin–streptomycin solution and Hanks balanced salt solution (HBSS) were bought from Gibco (Thermo Fisher Scientific, USA). Male rat liver microsomes and pooled human liver microsomes were purchased from RILD Research Institute for Liver Diseases (Shanghai, China). Nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Roche Diagnostics (Shanghai, China). Bovine serum albumin, dextran T-40, dexamethasone and norepinephrine were purchased from Sigma–Aldrich (St Louis, MO, USA). HPLC-grade methanol and acetonitrile were bought from Merck Millipore (Billerica, MA, USA). The other reagents were of analytical grade. The distilled water was obtained using a Milli Q water purification system (Millipore Corporation, Billerica, MA, USA).

Fifteen male pathogen-free Sprague–Dawley rats (220–250 g) were purchased from the Experimental Animal Center of Southern Medical University (Guangzhou, China, certificate no. SCXK 2013–0217). Rats were housed under a 12 h light–dark cycle for 7 days, at a room temperature of 22 ± 2°C and humidity of 55 ± 5% before the experiments. They were not fed for 12 h before drug administration and fed again at 2 h after administration. Rats had free access to water during the experiments.

2.2 | Instrumentation and LC–MS/MS method

The LC–MS/MS analysis was performed using a Shiseido Nanospace 1312 HPLC system coupled with an AB Sciex 4000 Q Trap™. Data acquisition and quantification were conducted using Analyst 1.6 (AB Sciex, USA).

WA was analyzed using a Venusil MP C_{18} column (50 × 2.1 mm, 5 μm) at ambient temperature with a flow rate of 0.25 mL/min. The mobile phase A consisted of acetonitrile and water at ratio of 5/95 (v/v), and phase B was composed of acetonitrile and water, 95/5 (v/v). Both phases contained 10 mM ammonium acetate. In the LC gradient profile, the mobile phase B started at 30% (v/v) until 0.3 min and then linearly increased to 100% at 0.5 min; it was held at 100% from 0.5 to 2.4 min, and it was then brought back to 30% at 2.8 min. The entire running time was 4.0 min. The retention times of WA and IS were 1.9 and 2.2 min respectively.

MS/MS conditions were as follows: source temperature, 550°C; ion spray voltage, 5500 V; curtain gas, 15 psi; gas 1, 70 psi; gas 2, 60 psi; collision gas, medium; entrance voltage, 9 V; and dwell time, 120 ms. The ion pairs for the MRM were *m/z* 471.4 to 281.2 for WA, and *m/z* 529.6 to 451.5 for IS. The declustering potential was 100 and 130 V for WA and IS, respectively. The collision energy was set at 25 and 29 eV for WA and IS, respectively. Product ion mass spectra for WA (A) and IS (B) are shown in Figure S1.

2.3 | Calibration solutions and quality control samples

Stock solutions (1 mg/mL) used for the WA standard curve, WA quality control and IS were respectively dissolved in methanol. WA working solutions were obtained by serial dilution in methanol to obtain the concentrations from 1 to 10,000 ng/mL. Standard curve and quality control (QC) samples in plasma were prepared in a similar way using blank plasma. The final concentrations were 0.2, 0.4, 1, 5, 20, 100, 500, 1600 and 2000 ng/mL for WA. QC samples were at the concentrations of 0.4, 20 and 1600 ng/mL after mixing with the blank plasma. All working solutions, stock solutions, standard curve and QC samples were immediately stored at 4°C after their preparation.

2.4 | Sample preparation

A 100 μL aliquot of each of samples such as plasma, HBSS sample from transport studies, intestinal microflora solution or the perfusate, with 20 μL methanol and 50 μL IS (1 μg/mL), was added to a 2 mL centrifuge tube placed on an ice-water bath and then mixed by vortexing. An amount of 1.5 mL ethyl acetate was further added to the mixture under shaking for 15 min in an ice-water bath to achieve a better extraction recovery. After centrifugation at 14,000***g*** and 4°C for 5 min, 1.4 mL of the supernatant was transferred into a tube and subjected to evaporation under vacuum until dehydration. The obtained residue was reconstituted in 200 μL of the mobile phase, and 20 μL of each resulting solution was injected into the LC–MS/MS system for analysis.

2.5 | Method validation

According to the newly US Food and Drug Administration Guidelines on Bioanalytical Method Validation (US FDA, 2018), the method for detecting WA in rat plasma samples was validated through selectivity, linearity, lower limit of quantification, precision and accuracy, matrix effect, extraction recovery, stability and dilution.

2.5.1 | Selectivity

The selectivity was evaluated by comparing the LC–MS/MS spectrogram of six different sources of blank plasma samples with spiked matrix samples of WA or IS.

2.5.2 | Linearity and sensitivity

The calibration standard of WA in plasma samples was analyzed during three consecutive days and prepared in triplicate as described above. The standard curve was obtained by plotting the ratio of the sample peak area and IS peak area vs the concentration of the calibration standard. Linear regression analysis was used to determine the slope, intercept and correlation coefficient. The lower limit of quantification (LLOQ) was defined as the lowest concentration in which precision and accuracy should not exceed 20%.

2.5.3 | Accuracy and precision

Accuracy and precision were evaluated using the QC samples (low, medium and high concentration) in six duplicate samples, which were analyzed on the same day and on three consecutive days. Relative standard deviation (RSD) was used to evaluate the intra- and inter-day precision, and relative error (RE) was used to assess the accuracy. The acceptance values used for validation of RSD and RE were within ±15%.

2.5.4 | Extraction recovery and matrix effect

The extraction recovery of WA was determined by comparing the mean peak area of regularly prepared QC samples (low, medium and high concentration) with the mean peak area of the spike-after-extraction samples (blank plasma extracted with ethyl acetate and then spiked with standards) at the same concentrations in six replicates. The matrix effect was assessed by comparing the peak area of post-extraction samples spiked with analytes with the peak area obtained from the mobile phase spiked with analytes at the same concentration.

2.5.5 | Stability

The stability was evaluated by analyzing six replicates of WA in plasma samples (100 μL) at three QC levels under different conditions, such as after 4 h storage in ice-water (0°C), after three freeze–thaw cycles (−80 to 4°C) and after storage at −80°C for 15 days. The post-preparative stability of the plasma samples during storage in the autosampler at 4°C for 24 h was also investigated. Samples were considered stable if the average percentage concentration deviation was within 15% of the actual value.

2.5.6 | Dilution

Ultrahigh WA samples (8000 ng/mL) were prepared in plasma and then diluted to high QC (1600 ng/mL) using blank plasma. The diluted high QC was analyzed in six replicates.

2.6 | Pharmacokinetic study of WA in male rats

The oral bioavailability of WA was recalculated by pharmacokinetic study in male rats. Animal experiments were conducted according to the Regulations of Experimental Animal Administration from the State Committee of Science and Technology of the People's Republic of China. The ethical approval of the study was provided by the ethics committee of the Institutional Animal Care and Use Committee of ZhongShan Pharmass Corporation [approval in Figure S5; license no. SYXK(粤)2018-0127 in Figure S6]. The WA solution (1.7 mg/mL for intravenous and intragastric administration) was prepared by diluting WA in a mix of ethanol–solutol HS 15–distilled water (10:5:85, v:v:v) (González, Huwyler, Walter, Mountfield, & Bittner, 2002; Hou et al., 2016; Kim et al., 2017). Two groups of male Sprague–Dawley rats ($n = 6$) were treated with an intravenous injection of 5 mg/kg WA or an oral administration of 10 mg/kg WA. A blood sample of ~300 μL was collected from each rat ophthalmic venous plexus and placed into heparinized tubes on an ice-water bath. Blood collection was performed at 0 (predose), 0.03, 0.17, 0.5, 1, 2, 4, 7, 12 and 24 h in the case of intravenous administration, and 0.83, 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h in the case of oral administration.

Blood samples were centrifuged at 3000***g*** and 4°C for 10 min to obtain the plasma that was stored at −80°C until analysis. To quantify WA in the plasma, the protocol described in Section 2.4 was used to

prepare the LC–MS/MS samples. The pharmacokinetic parameters were calculated using DAS software (version 3.0, Shanghai, China)

2.7 | Caco-2 cell monolayer

The intestinal permeability of drugs was a key factor affecting oral absorption. The Caco-2 cell line is a continuous cell line of heterogeneous human epithelial colorectal adenocarcinoma, widely used to evaluate drug permeability and used in transport assays (Fogh, 1975). Our Caco-2 cells were obtained from the Shanghai Institute of Cell Biology, the Chinese Academy of Sciences (Shanghai, China). They were routinely maintained in DMEM (high glucose) containing 10% fetal bovine serum, 400 mM L-glutamine, 4500 mg/L glucose, 100 mg/mL penicillin and streptomycin and incubated at 37°C under a 5% carbon dioxide and 90% relative humidity environment.

2.7.1 | WA toxicity assay in Caco-2 cells

Before any experiment, WA toxicity on Caco-2 cells was evaluated by MTT assay. Briefly, Caco-2 cells were seeded in 96-well plates with a density of 4000 cells per well. Twenty-four hours later, cells were treated with increasing concentrations of WA (1–100 μM) and incubated for 2 h. HBSS was used as a negative control. At the end of the incubation time, the MTT assay was performed according to the manufacturer's protocol (Beyotime), and the absorbance was measured by a spectrophotometer at 490 nm.

2.7.2 | WA transport assay in Caco-2 cells

WA transport across Caco-2 cells was evaluated according to a method previously reported with slight modifications (Lee et al., 2009). Cells at passages 20 and 40 were seeded on transwell polycarbonate insert filters (0.33 cm^2 surface, EMD Millipore Corporation). After 21 days of incubation the proper formation of a monolayer was verified by measuring the trans-epithelial electrical resistance. Drug transport from the apical side to the basolateral side (A–B) and from the basolateral side to the apical side (B–A) was measured under the same conditions in both directions. Digoxin was used as a positive control for P-glycoprotein (P-gp) mediated drug efflux. The assay was conducted using WA at concentrations of 2, 5 and 10 μM for 1 h. In brief, after the monolayer was formed, it was washed with HBSS three times, and WA diluted with HBSS was added to the appropriate well (final 0.1% ethanol, pH 6.8 for the apical side and pH 7.4 for the basolateral side). The plate was incubated at 37°C for 60 min. Samples were collected from both sides.

The concentration of WA in the samples was measured following the protocol described in Section 2.4 using LC–MS/MS. Samples with concentration over ULOQ were diluted 10-fold using the blank HBSS. The apparent permeability (P_{app}) was calculated using the following equation: $P_{app} = (\Delta Q/\Delta t)/C_0 A$, where $\Delta Q/\Delta t$ is the rate of permeation, C_0 is the initial concentration and A is the monolayer area (0.33 cm^2). As regards the bidirectional permeability, the efflux ratio was defined as P_{app}(B–A)/P_{app}(A–B); high efflux ratio (>2) indicates that a compound is a potential substrate for P-gp or other active transport system (Gao, Hugger, Beck-Westermeyer, & Borchardt, 2001; Li et al., 2016).

2.8 | WA stability in solutions at different pH along the gastrointestinal tract

Because some drugs (ester-containing drugs such as simvastatin or erythromycin) are prone to hydrolysis under strong acidic conditions, the stability of WA in the gastrointestinal pH should be evaluated. The stability of WA under a different pH environments was tested by incubating 100 μM WA at 37°C in a simulated rat gastric fluid (pH 2), human gastric fluid (pH 1.6) and HBSS buffer (pH 7.4) in triplicate (Jantratid et al., 2009; Willmann, Thelen, Becker, Dressman, & Lippert, 2010). A 100 μL aliquot of each incubation solution was collected at 0, 1 and 3 h and diluted 100-fold at each corresponding pH using the blank solution. The resulting solutions were further and immediately prepared for LC–MS/MS analysis according to Section 2.4. The ratios between WA and IS detected intensities were used to evaluate the stability of WA.

2.9 | WA stability in male rat and human intestinal microflora

Since drugs administered orally are inevitably in contact with a large amount of intestinal microflora in the intestinal tract, and WA is metabolized by several types of microbes, WA stability in male rat or human intestinal microflora was investigated (Funska, Khandlová, Šturdíková, Rosazza, & Kieslich, 1985; Fuska, Proska, Williamson, & Rosazza, 1987). The stability assay was performed according to a previous method (Chang, Han, Han, & Kim, 2009; Chang & Nair, 1995) with slight modifications. Fresh rat fecal samples (2 g in total) were obtained from six male Sprague–Dawley rats (230–250 g) and they were pooled and mixed with 20 mL sterile phosphate buffer (pH 7.4) in a sterile centrifuge tube. The mixture was centrifuged at 200***g*** and 4°C for 10 min, and then the resulting supernatant was collected and centrifuged at 9000***g*** at 4°C for 10 min. The obtained precipitate was resuspended in 40 mL of sterile phosphate buffer 20 mM (pH 7.4) to produce the rat intestinal microflora solution.

WA (100 μM) was anaerobically incubated in the above rat intestinal microflora solution (containing 1.5% ethanol) at 37°C in triplicate. One hundred microliters of the incubation solution were collected at 0, 0.25, 0.5, 1, 2, 4, 6, 12, and 24 h and diluted 100-fold with the blank rat intestinal microflora solution. The resulting solutions were further and immediately prepared for LC–MS/MS analysis according to the protocol described in Section 2.4. Parallel reactions without alive microflora (under sterilizing conditions at 115°C for 30 min) were conducted as negative controls; in contrast, the metabolism of rhoifolin (100 μM) in the rat intestinal microflora was evaluated and used as positive control (Jiang, Xiong, Dai, & Deng, 2016). The ratios between WA and IS detected intensities at each time point were compared.

Fresh human fecal samples (2 g in total) were obtained from six healthy Chinese volunteers (23–31 years, three males and three females) from ZhongShan Pharmass Corporation (Zhongshan city, Guangdong province, China) according to a protocol approved by Ethics Committee of ZhongShan Pharmass Corporation. They were processed as described above to evaluate the stability of WA in human intestinal microflora solution in triplicate.

2.10 | WA stability in male rat and human liver microsomes

Drugs that are absorbed through the gastrointestinal wall enter the liver from the portal vein for the first metabolism before going into systemic circulation. Therefore, the depletion rate of WA in male rat or human liver microsomes might affect the oral exposure. Since male Sprague–Dawley rats were used in the pharmacokinetic studies, male rat liver microsomes were used in the *in vitro* experiments. The two doses 1 and 5 μM WA were incubated with male rats liver microsomes or pooled human liver microsomes (both 0.2 mg/mL) in sodium phosphate buffer (0.1 M, pH 7.4) in triplicate. The reaction started by adding NADPH (1 mM) and stopped by transferring 100 μL reaction solution into ice-cooled 300 μL acetonitrile containing 50 ng/mL of IS at the reaction time of 0, 5, 15 and 35 min. Once stopped, the reaction mixtures were centrifuged (14,000***g***, 30 min, 4°C) and the supernatant was analyzed by LC–MS/MS. Depletion rate (*k*) and half-life were calculated using DAS software (version 3.0, Shanghai, China).

2.11 | Identification of the major WA metabolites *in vitro*

The identification of the main WA metabolites allowed it to be established whether the oral bioavailability of WA was reduced by the first-pass metabolism. WA at the concentration of 50 μM was incubated with male rat liver microsomes or pooled human liver microsomes (both 0.5 mg/mL) in sodium phosphate buffer (0.1 M, pH 7.4) at 37°C for 50 min. The incubation was initiated by adding NADPH (1 mM) and stopped by adding 2 volumes of ice-cooled acetonitrile containing 50 ng/mL IS. Parallel incubations terminated at 0 min were used as negative controls without metabolites for comparison. After centrifugation (14,000***g***, 30 min, 4°C) the supernatant was analyzed by Q-trap MRM–IDA–EPI analysis.

The function of Q-trap is to enrich the ions in the linear trap and enhance the intensity of fragments, and the analysis was performed as follows: first, the expected ion pairs for the unknown metabolites were predicted by Analyst™ 1.6 (AB Sciex, USA). To be specific, the software predicted the ion pairs based on the known parent and daughter ions of WA (471, 281, and 299, as shown in Figure S1). According to the existing metabolic types, for example, $M + O + H^+$, if as a result of the metabolism an oxygen was added to WA, the parent ion would be 487 (471 + 16 = 487). Then, an oxygen could be added to the daughter ions 297 (281 + 16 = 297) and 315 (299 + 16 = 315) or not added (281, 299), depending on the potential metabolic site. Finally, four ion pairs of $M + O + H^+$ (487–281, 487–297, 487–299 and 487–315) were predicted.

If the intensity of the predicted metabolite ion exceeds 5000 cps, it triggers the EPI scan to obtain higher signal of the metabolite's fragments (Sun et al., 2015). The samples incubated for 50 min were compared with the negative control to exclude interference ions. Fragments of metabolites were compared with the fragments of WA for further confirmation.

WA and its metabolites were analyzed on a Simpsil C_{18} (250 mm × 4.6 mm, 5 μm) at ambient temperature with a flow rate of 0.3 mL/min. Mobile phase A was composed of acetonitrile and water at a ratio of 5:95 (v/v), and B was composed of acetonitrile and water, 95:5 (v/v). Both phases contained 0.1% formic acid. In the LC gradient profile, the mobile phase B was 85% (v/v) until 0.3 min and then linearly increased to 100% from 0.3 to 1 min, remaining at 100% from 1 to 16 min, and it was then brought back to 85% from 16 to 17 min. The entire running time was 25 min. Mass conditions were as follows: source temperature, 300°C; ion spray voltage, 5500 V; curtain gas, 15 psi; gas 1, 70 psi; gas 2, 60 psi; collision gas, medium; entrance potential, 9 V; and dwell time, 30 ms. The collision energy (CE) was set at 30 eV with a spread of 10 eV and the scan rate was 1000 Da/s.

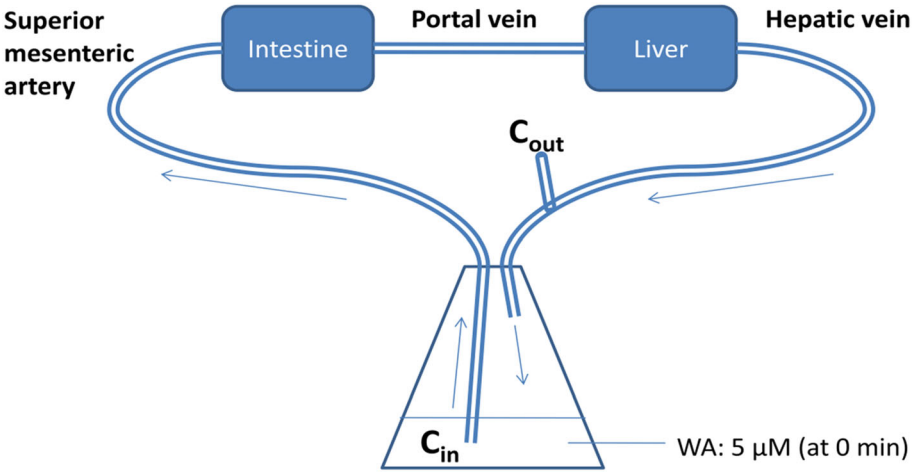

FIGURE 1 The schematic diagram of *in situ* perfused rat intestine–liver preparation

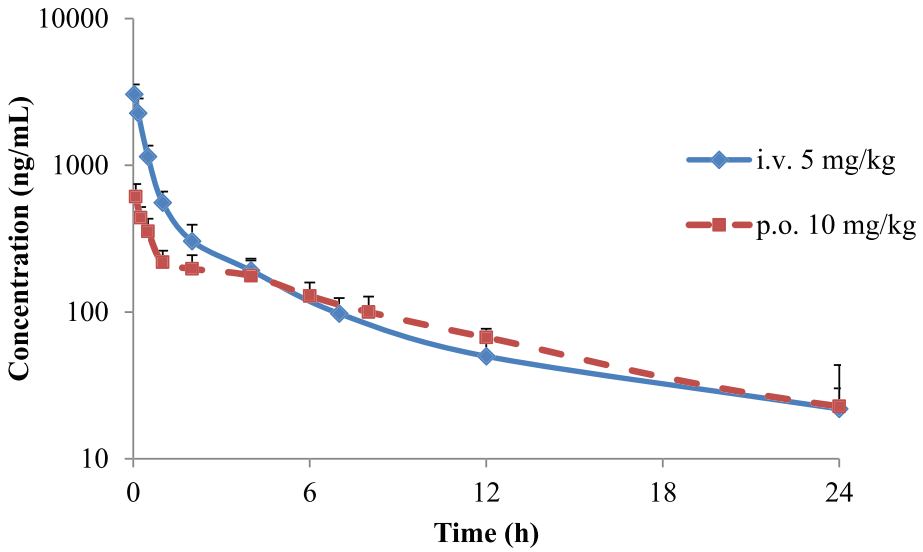

FIGURE 2 Mean plasma concentration–time profiles of withaferin A (WA) in rats ($n = 6$)

2.12 | *In situ* rat intestine–liver perfusion

In situ rat intestine–liver perfusion allowed verification of whether the first-pass metabolism resulted in reduction of oral bioavailability of WA. The perfusate was composed of a Krebs–Ringer solution containing 5% washed rat red blood cells, 0.3% glucose, 1% bovine serum albumin, 3% dextran T-40, 0.02% dexamethasone and 0.004% norepinephrine (Wang et al., 2012). A final concentration of 5 μM WA was added into the perfusate and mixed at the beginning of the perfusion. Three male Sprague–Dawley rats of ~220–250 g were fasted for 12 h before surgery. The surgical procedure for *in situ* rat intestine–liver perfusion was performed based on a method previously described (Pang, Cherry, & Ulm, 1985). After ligation, the blank perfusate (at 37°C without red blood cells and WA) was infused from the superior mesenteric artery to be released from the hepatic vein to exclude the presence of remaining blood in the intestine and liver, as shown in Figure 1. Then 5 μM WA in the reservoir (37°C, 95% O_2, 5% CO_2) was pumped into the perfusate system with a flow rate of 7.5 mL/min. The outflow was conveyed back to the reservoir. Each 300 μL perfusate in the reservoir (C_{in}) or in the hepatic venous outflow (C_{out}) were collected at 0, 1, 5, 10, 20, 30, 45 and 60 min, and they were immediately centrifuged at 3000***g*** for 10 min at 4°C. The resulting supernatants were prepared for analysis according to the protocol described in Section 2.4. The intestine–liver first-pass effect of WA was calculated in the concentration–time curve as follows: $(AUC_{in} - AUC_{out})/AUC_{in} \times 100\%$ (AUC, area under the concentration–time curve of WA). The metabolites were also analyzed, and the ratio between metabolite and IS relative intensity at each time point was calculated.

TABLE 1 Noncompartmental pharmacokinetic parameters of withaferin A (WA) after intravenous administration of 5 mg/kg or oral administration of 10 mg/kg WA in rats ($n = 6$, mean ± SD)

	Rat	
Parameter	Intravenous	Oral
AUC_{0-t} (ng/mL h)	3615 ± 670	2345 ± 345
$AUC_{0-\infty}$ (ng/mL h)	3685 ± 685	2789 ± 683
MRT_{0-t} (h)	4.0 ± 0.4	6.8 ± 1.1
$MRT_{0-\infty}$ (h)	4.5 ± 0.8	10.8 ± 4.5
$t_{1/2}$ (h)	4.5 ± 1.1	7.6 ± 3.3
T_{max} (h)	–	0.11 ± 0.07
CL (L/h/kg)	1.4 ± 0.3	3.9 ± 1.1
V (L/kg)	9 ± 2	41 ± 15
C_{max} (ng/mL)	3048 ± 509	619 ± 125
F (%)	–	32.4 ± 4.8

AUC, Area under the concentration–time curve; MRT, mean residence time; $t_{1/2}$, half-life; T_{max}, time to peak concentration; *CL*, clearance; *V*, volume of distribution; C_{max}, peak concentration; *F*, bioavailability.

TABLE 2 Apparent permeability coefficients (P_{app}) of WA and digoxin across the Caco-2 cell monolayers (mean ± SD, $n = 3$)

Compound	Concentration (μM)	P_{app} (10^{-6} cm/s)		Efflux ratio
		A–B	B–A	
Withaferin A	2	13.7 ± 0.3	5.6 ± 0.8	0.41
Withaferin A	5	11.1 ± 0.8	5.2 ± 0.5	0.46
Withaferin A	10	10.2 ± 2.1	5.4 ± 0.4	0.53
Digoxin	10	1.8 ± 0.2	10.6 ± 0.5	6.00

TABLE 3 The stabilities of WA at different pH in triplicate (%)

Time (h)	pH = 1.6	pH = 2	pH = 7.4
0	100 ± 6	100 ± 8	100 ± 1
1	101 ± 6	96 ± 9	92 ± 6
3	104 ± 2	104 ± 4	110 ± 2

After the perfusion at 60 min, the liver, small intestine, large intestine and their intestinal contents were weighed and homogenized, and the resulting solutions were prepared according to Section 2.4 to quantify WA. The recovery of WA was calculated by the following formula: $(V_{60min} * C_{in\ 60min} + \text{organs})/(V_{0min} * C_{in\ 0min}) \times 100\%$ (where V is the volume of the perfusate in the reservoir, 'organs' means the amount of WA in the above organs).

3 | RESULTS AND DISCUSSION

3.1 | Optimization and validation methods

These methods were successfully applied to the *in vitro* measurement for WA and the pharmacokinetic studies in male rats. A better extraction of WA using ethyl acetate rather than *tert*-butyl methyl ether was obtained with a LLOQ of 0.2 ng/mL (Patil et al., 2013). Drugs in plasma samples were tested after extraction with *tert*-butyl methyl ether, ethyl acetate or ethyl ether. The results showed that the extraction recovery was the highest after ethyl acetate extraction. The LC condition was optimized using different concentrations of ammonium acetate or formic acid, and 10 mmol/L ammonium acetate was applied.

Results of the validation are shown in Tables S1–S5. Selectivity is shown in Figure S2. No interference was found. The calibration curve of WA was linear over the range 0.2–2000 ng/mL in plasma giving a correlation coefficient (r^2) of 0.99. The LLOQ was 0.2 ng/mL (accuracy and precision were 106.7 and 3.8%, respectively). The intra- and inter-day precisions at QC concentrations were <15% with accuracies ranging from 88.9 to 102.8%. The recovery of WA varied from 84.7 to 97.1% and the matrix effects were from 94.% to 104.8%. WA was stable in plasma for 4 h in ice-water (0°C), after

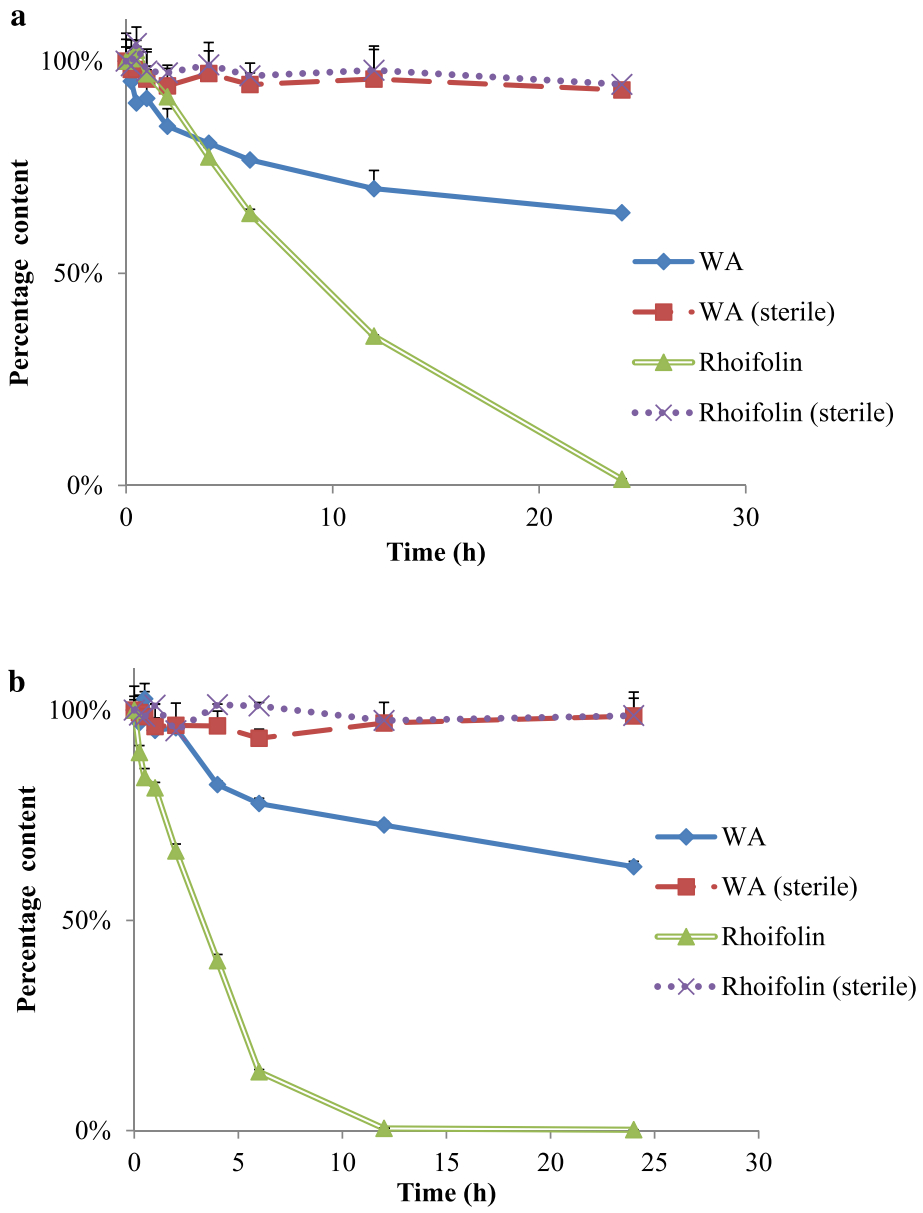

FIGURE 3 Percentage content of remaining WA and rhoifolin–time profiles in (a) rat intestinal microflora, (b) human intestinal microflora, (n = 3)

three freeze–thaw cycles, and for 15 days at −80°C. The post-preparative samples during storage in the autosampler at 4°C were also stable for 24 h. The relative standard deviation of the stability samples was <14%. The dilution results indicated that samples could be linearly diluted. The above results were within an acceptable range in plasma samples.

However, WA was not stable in rat plasma at room temperature, so both sample collection and pre-treatment were performed in an ice-water bath. The stability of WA in rat plasma at 0°C and 25°C for 4 hours are shown in Figure S3. At 0°C, the extraction recovery of the sample was higher than 90% for at least 4 hours.

3.2 | Pharmacokinetic study

The pharmacokinetic mean plasma concentration–time profiles of WA after intravenous (5 mg/kg) or oral (10 mg/kg) administration are shown in Figure 2. The noncompartmental pharmacokinetic parameters are shown in Table 1. The absolute oral bioavailability (F) value of WA was 32.4 ± 4.8% obtained by the following formula: $F = [(AUC_{oral}/AUC_{intravenous}) \times (Dose_{intravenous}/Dose_{oral})] \times 100\%$. This oral bioavailability in male rats was higher than the 9% estimated in mice, the latter appearing as an underestimation of the oral bioavailability of WA in mice (Ku et al., 2014; Patil et al., 2013). Thus, it is necessary to further improve absorption and therapeutic efficacy by the *in vitro* identification of the potential factors affecting oral bioavailability in rats and humans.

3.3 | Transport of WA across Caco-2 monolayer

Before the transport experiment, WA cellular toxicity was examined by an MTT assay, and the results showed that 99% of the cells survived after treatment with 10 μM WA for 2 h compared with the negative control. The trans-epithelial electrical resistance value was 410 ± 32 Ω cm^2. To verify the efflux activity of P-gp, a typical P-gp substrate, digoxin, was used as the positive control. The efflux ratio of digoxin of 6.00 indicated that the efflux activity of P-gp was appropriate for this experiment. Furthermore, both WA and digoxin showed high recovery from the transport study (recovery from 92 to 107%).

Table 2 shows the permeability of WA across Caco-2 cell monolayers. The P_{app} of WA from the A–B side was 1.37×10^{-5}, 1.11×10^{-5} and 1.02×10^{-5} cm/s at the concentrations 2, 5 and 10 μM, respectively, which were all $>1.0 \times 10^{-5}$ cm/s, indicating that WA could be easily transported across Caco-2 cells in the absorption direction (Gao et al., 2001). This good permeability in Caco-2 cells was the opposite of the results previously reported on an MDCK cells system (Devkar et al., 2015). Although Caco-2 and MDCK cells are predictors for permeability, the discrepancy between the results of Devkar *et al*. and our results could be potentially attributed to species difference or different expressions of transporters. Indeed, Caco-2 is from human colorectal adenocarcinoma containing many different types of transporters, but MDCK is from canine kidney, giving a clear transporter background (The International Transporter Consortium, 2010). Therefore, passive diffusion as a major pathway could be determined using MDCK. In contrast, it comes with the mix contribution including the passive diffusion and transporters pathways when using the Caco-2 system. Additionally, contradictory results were observed in the transport of other compounds with high permeability in Caco-2 cells (P_{app} $>1.0 \times 10^{-5}$ cm/s), but with low permeability in MDCK cells (P_{app} $<2.0 \times 10_{-6}$ cm/s), similar to the contradiction observed with WA (Jin et al., 2014).

As regards P-gp, it is extensively distributed and expressed not only in rat and human intestinal epithelium, but also in the endothelial cells of the blood–brain barrier capillaries (Cao et al., 2006; Stephens et al., 2001). In the present study, the B–A P_{app} of WA was lower than the A–B P_{app} and the calculated efflux ratios at different WA concentrations were all <2, as shown in Table 2, suggesting that WA was not a substrate for P-gp or other intestinal efflux transporters (Li et al., 2016). As a result, WA might be easily transported in both human

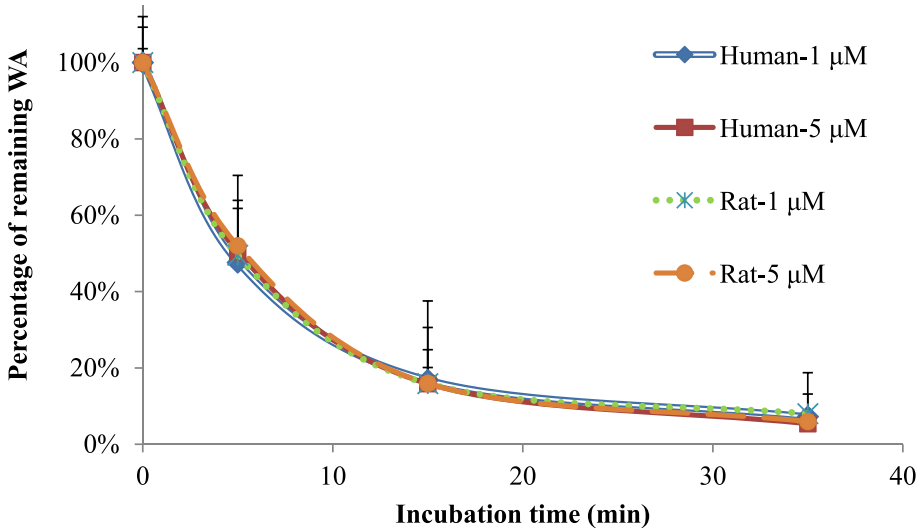

FIGURE 4 Percentage content of remaining WA–time profiles of 1 μMand 5 μM WA in rat or human liver microsomes ($n = 3$)

and rat intestinal cells, since a permeability correlation of $r^2 = 0.8$ was observed based on the results from *in situ* intestinal perfusion in rats compared with the *in vivo* human jejunal perfusion for 14 drugs (Cao et al., 2006).

3.4 | Stability of WA at different pH

Although there are lactones and an epoxy group in WA, Table 3 shows that WA was stable in simulated gastric fluids at pH 2 (rat) and pH 1.6

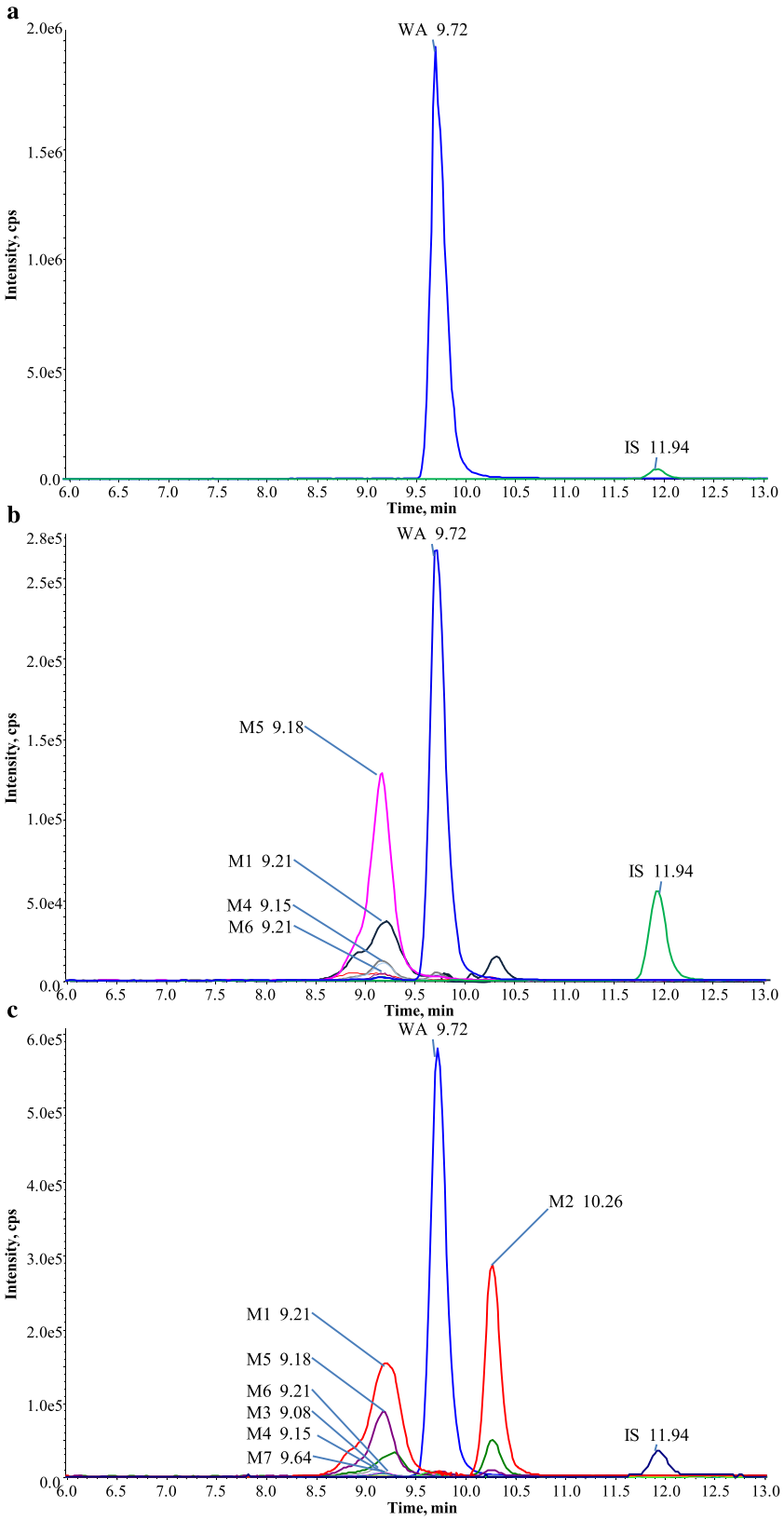

FIGURE 5 The total ion chromatographS (TIC) of predicted metabolite ion pairs in multiple reaction monitoring (MRM) mode for the control (a), rat liver microsomes (b) and human liver microsomes (c)

(human) and in HBSS buffer (pH 7.4) within 3 h, unlike other lactones such as erythromycin and simvastatin.

3.5 | Stability of WA in male rat and human intestinal microflora

It has been reported that WA can be metabolized by several types of microbes and we also found a similar slow conversion of WA by the intestinal microflora of male rats and humans (Funska et al., 1985; Fuska et al., 1987).

Figure 3 shows that the positive control rhoifolin (100 μM) was rapidly eliminated in male rat and human intestinal microflora solution and its content was <2% at 24 h. In contrast, the content of WA (100 μM) decreased slowly, remaining as high as 64.3% and 62.8% at 24 h in male rat and human intestinal microflora solution, respectively, revealing that the conversion of WA by intestinal microflora was slow. Moreover, the depletion of WA was <15% within 2 h in these intestinal microflora solutions, while the T_{max} in the oral administration studies performed in male rats was 0.11 h, suggesting that this intestinal microflora might have little influence on the rapid absorption of WA.

3.6 | Stability of WA in male rat and human liver microsomes

To evaluate the metabolism of WA in male rat live microsomes and human liver microsomes, the depletion rate of WA was detected at the substrate concentration of 1 μM (which approached the peak concentration, C_{max}, in the oral administration study in male rats) and 5 μM (Figure 4). The depletion rate of WA in male rat liver microsomes was close to that in the human liver microsomes at the same substrate concentration and a microsome protein concentration of 0.2 mg/mL (k_{rat} = 0.124 min^{-1}, k_{human} = 0.122 min^{-1}). The metabolism of WA in both male rat and human liver microsomes was rapid, with a half-life of ~5.6 min. Therefore, the rapid metabolism of WA in liver microsomes might be a critical aspect influencing the oral bioavailability and it requires further elucidation to evaluate whether the first-pass effect is involved in the oral absorption of WA (Takahashi, Washio, Suzuki, Igeta, & Yamashita, 2009).

3.7 | Identification of the major metabolites of WA *in vitro*

The rapid metabolism of WA (half-life = 5.6 min) both in male rat and human liver microsomes led to a large production of different metabolites. In this work, seven metabolites in human and four in male rat microsomes were found.

The total ion chromatography in MRM mode of the control group (a), the male rat liver microsomes samples (b), and human liver microsomes samples (c) are shown in Figure 5. The ion pair, which exceeded 5000 cps in MRM mode, was simultaneously scanned in EP) mode to acquire fragments of the corresponding precursor ions (Figure 6).

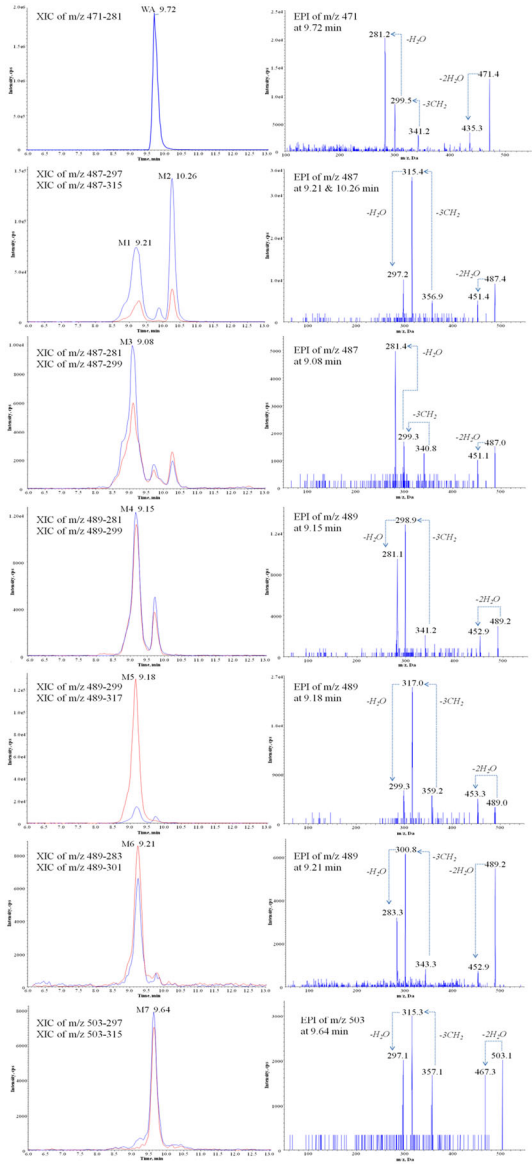

FIGURE 6 The extracted ion chromatograms and the major fragment ions of WA and 7 proposed metabolites in enhanced product ion (EPI) mode

Table 4 and Figure 6 show WA and its seven proposed metabolites with similar fragmentation patterns. The base peak of WA at *m/z* 281 corresponds to the remaining left part of the molecule after losing the lactone ring, ethyl group and one water molecule. According to the fragmentation pattern of WA, both the two major fragment peaks of WA, 281 and 299 possessed obvious characteristic peaks of oxygenation (+16), hydrolysis (+18) and oxygenation–hydrogenation (+18) in the fragment peaks of its metabolites, such as 297, 315 and 317. Another two fragment peaks of WA, 341 and 435 also showed the same pattern in its metabolites, such as 357, 359, 451 and 453.

Hence, the MRM–IDA–EPI method was employed to explore the metabolites and found these major metabolites via hydroxylation, hydrogenation and hydrolysis of the parent compound in liver microsomes (Figure 7). Among these seven identified metabolites, M2, M3

TABLE 4 The major fragments and retention time of WA and its seven proposed metabolites detected in RLM and HLM

Compound	Composition	Precursor ion (*m/z*)	Major fragments (*m/z*)	Retention time (min)	RLM	HLM
WA	$M+H^+$	471	281*, 299, 341, 435	9.72	+	+
M1	$M+O+H^+$	487	297, 315*, 357, 451	9.21	+	+
M2	$M+O+H^+$	487	297, 315*, 357, 451	10.26	-	+
M3	$M+O+H^+$	487	281*, 299, 341, 451	9.08	–	+
M4	$M+H_2O+H^+$	489	281, 299*, 341, 453	9.15	+	+
M5	$M+O+2H+H^+$	489	299, 317*, 359, 453	9.18	+	+
M6	$M+O+2H+H^+$	489	283, 301*, 343, 453	9.21	+	+
M7	$M+O+O+H^+$	503	297, 315*, 357, 467	9.64	–	+

*The base peak in the EPI mode.

RLM, Rat liver microsomes.

HLM, Human liver microsomes.

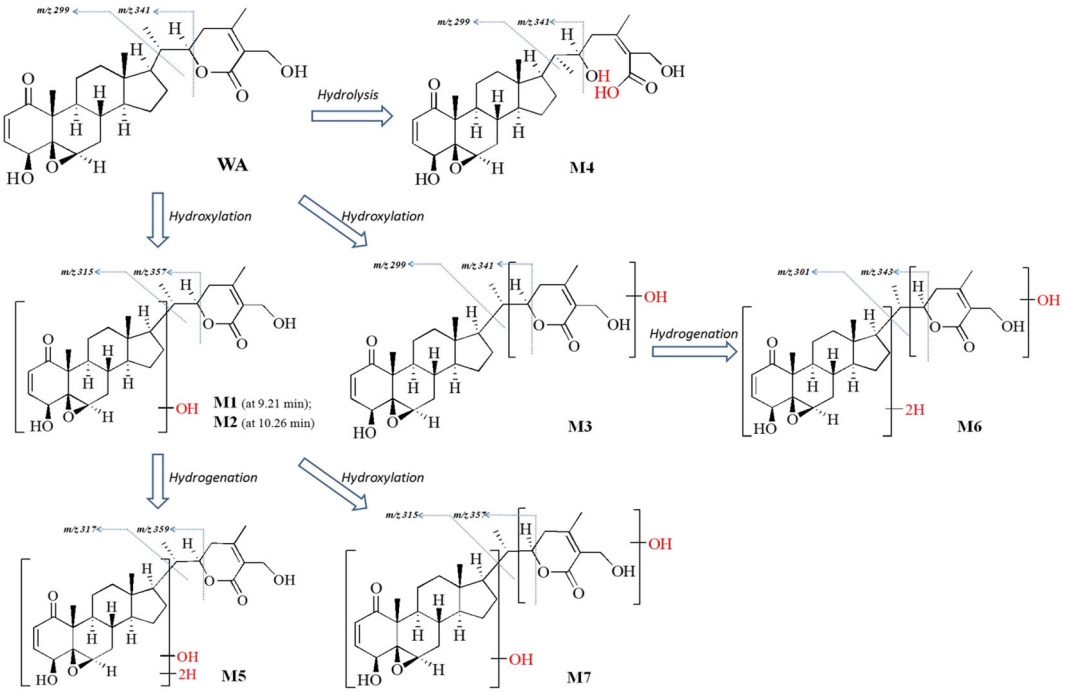

FIGURE 7 The proposed metabolic pathways of WA in rat or human liver microsomes and the their fragmentation patterns

and M7 were specifically found in human. The neutral loss and precursor ion scan mode combined with EPI scan were also used to find additional metabolites, but no other metabolites were found. These methods cannot exclude the possibility that other major metabolites might be generated through other metabolic pathways, especially by the phase II metabolizing enzymes.

3.8 | *In situ* rat intestine–liver perfusion

Considering the significant correlation between the metabolic rate in liver microsomes and hepatic first-pass metabolism (Takahashi et al., 2009), the first-pass metabolism was assessed by the *in situ* rat intestine–liver perfusion at the same concentration of 5 μM as used in microsomes, resulting in a similar decrement to 1.2 μM (near C_{max}) during perfusion as the *in vivo* study, as shown in Figure 8.

A small change (<5%) in the volume of the perfusate during the perfusion was found, while no significant organ edema was observed. Figure 8 shows that the concentration of WA in the inflow was much higher than its concentration in the outflow at the beginning of the perfusion and then became closer with time. The intestine–liver first-pass effect of WA was calculated to be 72.9 ± 6.5%, suggesting that most of the WA might be biotransformed or bound to the tissue after passing through the intestinal wall and liver. The rest of WA that entered into the systemic circulation accounted for ~27.1%, which was consistent with the oral bioavailability of 32.4 ± 4.8% found *in vivo*. Furthermore, M1, M4 and M5 metabolites were detected in the perfusate in an increased content, suggesting that hydroxylation,

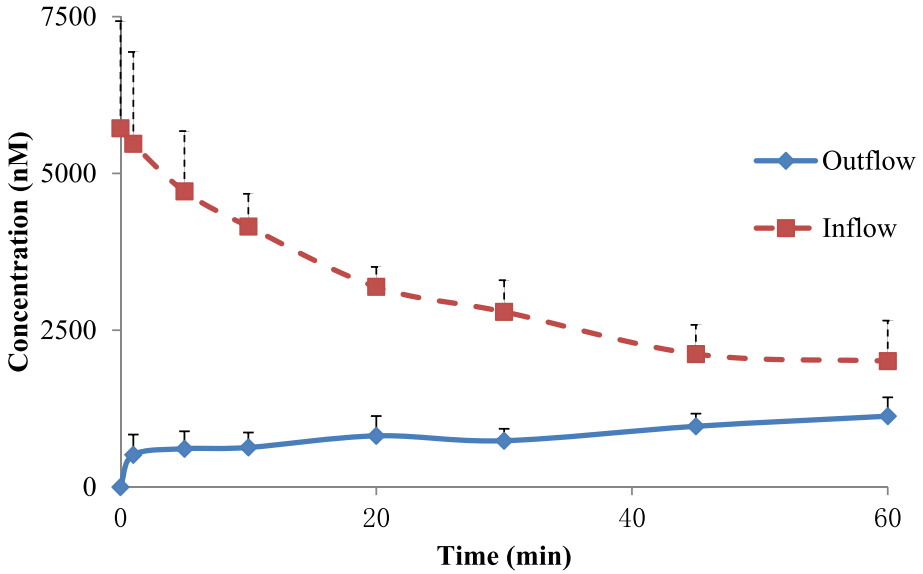

FIGURE 8 The perfusate concentration–time profiles of WA at the inflow (superior mesenteric artery), and at the outflow (hepatic venous) *in situ* rat intestine–liver perfusion ($n = 3$)

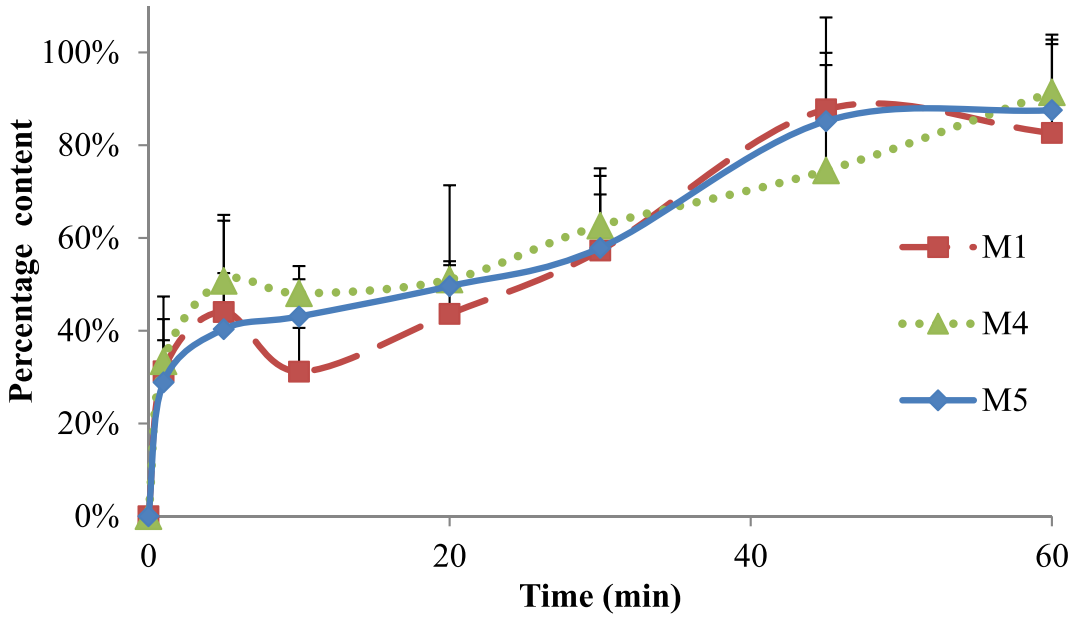

FIGURE 9 The percentage content–time profiles of proposed metabolites M1, M4 and M5 detected at the outflow (hepatic venous) in situ rat intestine–liver perfusion ($n = 3$)

hydrogenation and hydrolysis of WA happened in the intestine and liver (Figure 9). In addition, M1, M5 and possible glucuronide conjugates were detected in the liver, small intestine and large intestine.

The concentration of WA in organs is shown in Figure S4, while no drug was found in the content inside the intestine. The total recovery of WA was 22.2%, including only 2.9% WA bound to the organs and another 19.3% remained in the perfusate solution at 60 min. As a result, ~77.8% of total WA might be metabolized.

To reduce the first-pass metabolism, WA may be supplied by rectal administration. Using this route of administration, drugs penetrate the rectal mucosa and enter the iliac vein, then into the inferior vena cava, thus avoiding the hepatic first-pass metabolism and increasing the bioavailability by noninvasive administration routes (Dhillon, Oxley, & Richens, 2012; Stoppelaar de, Stolk, Beysens, Stappers, & Gorgels, 1999).

4 | CONCLUSION

The oral bioavailability of WA was 32.4 ± 4.8% in male rats. In order to further improve the absorption and therapeutic efficacy of WA, the

potential factors affecting the oral bioavailability were identified and compared between rats and humans by *in vitro* screenings.

In vitro, WA could be easily transported across Caco-2 cells and the stability of WA was similar between male rat and human in simulated gastric fluid (stable) and in intestinal microflora solution (slow decrease). However, WA was rapidly removed either in male rat or in human liver microsomes, and further verification revealed that strong first-pass metabolism occurred in male rats. Hence, the first-pass metabolism of WA might be the main barrier in achieving good oral bioavailability in male rats and predicted in humans. Thus, the oral administration may reduce either the exposure and/or the therapeutic effect of WA in brain to combat glioma and Alzheimer's disease. Furthermore, it should be noted that, although WA and some compounds had high permeability, first-pass metabolism could still affect their absorption, especially for compounds that were rapidly eliminated in liver microsomes.

Our study on the *in vitro–in vivo* relationship for evaluating the absorption of WA in male rats, along with the understanding of the metabolic profile, depletion rate and permeability between rat and human *in vitro*, will provide an important insight into the utilization of preclinical and *in vitro* data to predict absorption-related factors.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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