

Development and validation of a LC–MS/MS method for determination of pinoresinol diglucoside in rat plasma: Application to pharmacokinetic study

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Abstract: Pinoresinol diglucoside (PD), a typical marker compound in *Ecommia ulmoides* Oliv., is an important and natural antihypertensive drug. A selective, sensitive, and rapid liquid chromatography tandem mass spectrometric (LC-MS/MS) analytical method was developed for the determination of PD in rats. After simple protein precipitation with acetonitrile, chromatographic separation of PD was conducted using a reversed-phase ZORBAX SB C₁₈ analytical column (4.6mm × 150mm, 5µm particles) with a mobile phase of 10mM ammonium acetate-methanol-acetic acid (50:50:0.15, v/v/v) and quantified by selected reaction monitoring mode under positive electrospray ionization condition. The chromatographic run time was 3.4 min for each sample, in which the retention times of PD and the internal standard were 2.87 and 2.65min, respectively. The calibration curves were linear over the range of 1.00–3000ng/mL and the lower limit of quantification was 1.00ng/mL in rat plasma. The precision expressed by relative standard deviations were <8.9% for intra-batch precision and <2.0% for inter-batch precision, and the intra- and inter-batch accuracy by relative error was within the range of -3.9% ~7.3%, which met acceptable criteria. The LC-MS/MS method was successfully applied to investigate the pharmacokinetics and oral bioavailability of PD in rats, with the bioavailability being only 2.5%.

Keywords: Pinoresinol diglucoside, oral bioavailability, LC-MS/MS, *Ecommia ulmoides*

INTRODUCTION

Tu-Chung, belonging to one of the well-known oldest herbs, has been used for many years in China (Sun *et al.*, 2006; Ye, 2004). The herb proved to have several effects of strengthening muscles and bones, and tonifying the liver and kidney as well as preventing miscarriage (Kwan *et al.*, 2004; State Pharmacopoeia Committee, 2010). Phytochemical investigation of the *Ecommia ulmoides* proved that several types of natural products were identified, such as flavonoids, iridoid glycosides, phenylpropanoids, polysaccharides and lignans (Deyama *et al.*, 2001; Guan and Su, 2003; Li *et al.*, 2012). Pinoresinol diglucoside (PD, fig. 1), mainly isolated from the herbal Tu-Chung (*Ecommia ulmoides* Oliv.) for many years, was definitely considered to be the prominent antihypertensive component of Tu-Chung material (Sih *et al.*, 1976; Zhao *et al.*, 2010). The antihypertensive activity of PD was mainly focused on the vasodilatation effect (Sun and Xu, 2006; Xu *et al.*, 2006), in which the mechanism might be related with the inhibition of voltage dependent calcium channel and activation of ATP sensitive potassium channel, as well as the mediated pathway by nitrogen monoxidum or prostacyclin (Zhou *et al.*, 2007).

A few methods for assaying PD in the herbal materials or

the preparations had been reported, such as high-performance liquid chromatography with ultraviolet detection (HPLC-UV) (Wu *et al.*, 2008; Zhang *et al.*, 2009), high-performance liquid chromatography with the coulometric electrode array detection (HPLC-CEAD) (Heinonen *et al.*, 2001), ultra-performance liquid chromatography with ultraviolet detection (UPLC-UV) (Zhao *et al.*, 2010; Zhang *et al.*, 2010), ultra-performance liquid chromatography with photodiode array (UPLC-PDA) (Song *et al.*, 2010), and near-infrared reflectance spectroscopy (NIRS) (Li *et al.*, 2010; Chang *et al.*, 2011). However, these methods are not compatible for the assay of PD in biological fluids, due to the disadvantages of poor sensitivity and long chromatographic time. Recently, Wang *et al.* developed an LC-MS/MS method to quantify PD in rat plasma following oral administration of *Ecommia ulmoides* extract (Wang *et al.*, 2012). However, the main drawbacks included larger plasma volume (0.1mL), and a complicated elution procedure with longer running time (20 min) when compared with our method. In this study, we are presenting a more rapid and simple LC-MS/MS method within 3.4 min using just 50µL plasma. The newly developed LC-MS/MS method was successfully applied to the absolute oral bioavailability (*F*) of PD following intravenous (iv) or oral administration in rats.

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MATERIALS AND METHODS

Reagents and chemicals

PD (purity, >98.0%) was obtained from Tauto Biotech Com. Ltd (Shanghai China). The standard catalpol was obtained from the National Institutes for Food and Drug Control (Beijing, China). HPLC-grade ammonium acetate and methanol were purchased from Tedia Com. (Fairfield, USA). Water was purified by double-distillation treatment and then filtered through a 0.22 μ m membrane filter before use. All other chemicals were of analytical grade for the experiment.

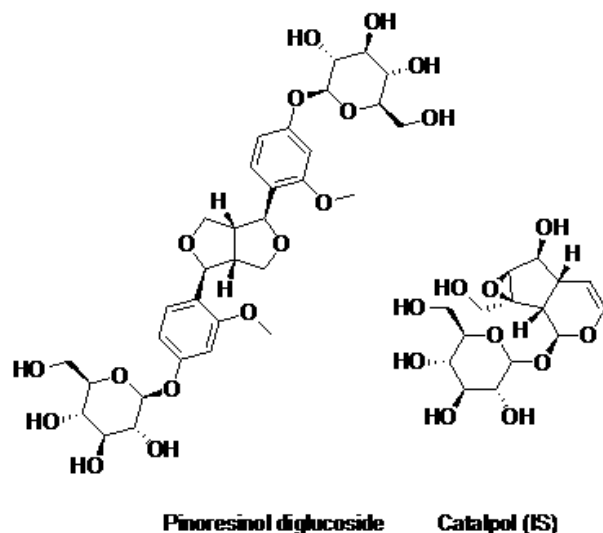


Fig. 1: Chemical structures of the pinoresinol diglucoside and catalpol.

Animals

Twelve male Wistar rats (Weighting 200 \pm 20g) were obtained from the Animal Center of Jilin University (Jilin, China). Rats were housed in a feeding room with a temperature-controlled (23 \pm 2 $^{\circ}$ C), humidity-controlled condition (45 \pm 10%), and a 12 h dark-light cycle. They had free access to food and water all the time. After housing under the above conditions for 7 days, the rats were fasted for 12 h before the experiments.

Instruments and chromatographic conditions

An Agilent 1200 series high-performance liquid chromatograph (HPLC) system and an Agilent 6460 triple-quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Agilent Technologies, USA) were applied for the analysis. The mobile phase consisted of 10mM ammonium acetate-methanol-acetic acid (50:50:0.15, v/v/v). An Agilent ZORBAX SB C₁₈ analytical column (4.6mm \times 150mm, 5 μ m particles) with a Phenomenon C₁₈ guard column (4.6mm \times 30mm, 5 μ m particles) was used for chromatographic separation. The column was equilibrated and eluted isocratically with a flow rate of 0.60mL/min, maintained at 30 $^{\circ}$ C. The injection volume was 20 μ L and 2084

the total run time was 3.4 min for each sample. Quantification was performed in positive selected reaction monitoring (SRM) mode at the transitions m/z 700.3 \rightarrow m/z 235.1 for PD and m/z 380.1 \rightarrow m/z 165.0 for the IS, respectively. The instrument parameters were set as follows: gas temperature, 350 $^{\circ}$ C; gas flow, 11.0L/min; nebulizer pressure, 45 psi; capillary voltage, 4.0 kV. The parameters were optimized such as fragmentor voltage, 135V for PD and IS; collision energy, 10eV for PD and 15eV for IS.

Standard solution and quality control (QC) sample preparation

Individual stock solution of PD (580 μ g/mL) and catalpol (IS, 1.00mg/mL) were prepared by dissolving each compound in methanol. Then several standard working solutions (10.0, 30.0, 100, 300, 1000, 3000, 10000 and 30000ng/mL) were gradually prepared by diluting the stock solution of PD with methanol. These working solutions were used for daily preparation of standard calibrators at concentrations of 1.00, 3.00, 10.0, 30.0, 100, 300, 1000 and 3000ng/mL in blank rat plasma. The quality control (QC) working solution was prepared in the same way as the standard working solutions, and QC samples (low, 3.00ng/mL; medium, 100ng/mL; and high, 2700ng/mL) were prepared by diluting the working solution with blank rat plasma. The working IS solution (catalpol, 5000ng/mL) was prepared by diluting the IS stock solution with methanol.

Sample preparation

200 μ L of methanol, and 50 μ L of IS solution (5000 ng/mL) were gradually added to 50 μ L of plasma sample. The mixture was sequentially vortexed for 5min and centrifuged at 12000rpm for 10min. Then 100 μ L of the supernatant was transferred to the autosampler vial and a 20 μ L of aliquot was injected into the LC-MS/MS system for analysis.

Method validation

The method validation was performed including selectivity, precision, accuracy, linearity, sensitivity, matrix effect, extraction recovery, stability and dilution integrity according to FDA bioanalytical guidelines (US DHHS, 2006).

Selectivity

The selectivity of the LC-MS/MS method was evaluated by comparing chromatograms of rat blank plasma samples, blank plasma spiked with standard and IS, and rat plasma sample after oral or iv administration of PD.

Precision and accuracy

The intra-day precision and accuracy were investigated by assaying six triplicates of QC samples of PD at low, medium, and high concentrations (3.00, 100 and 2700 ng/mL) on the same day. The inter-day accuracy and precision were performed by analysis of three batches of

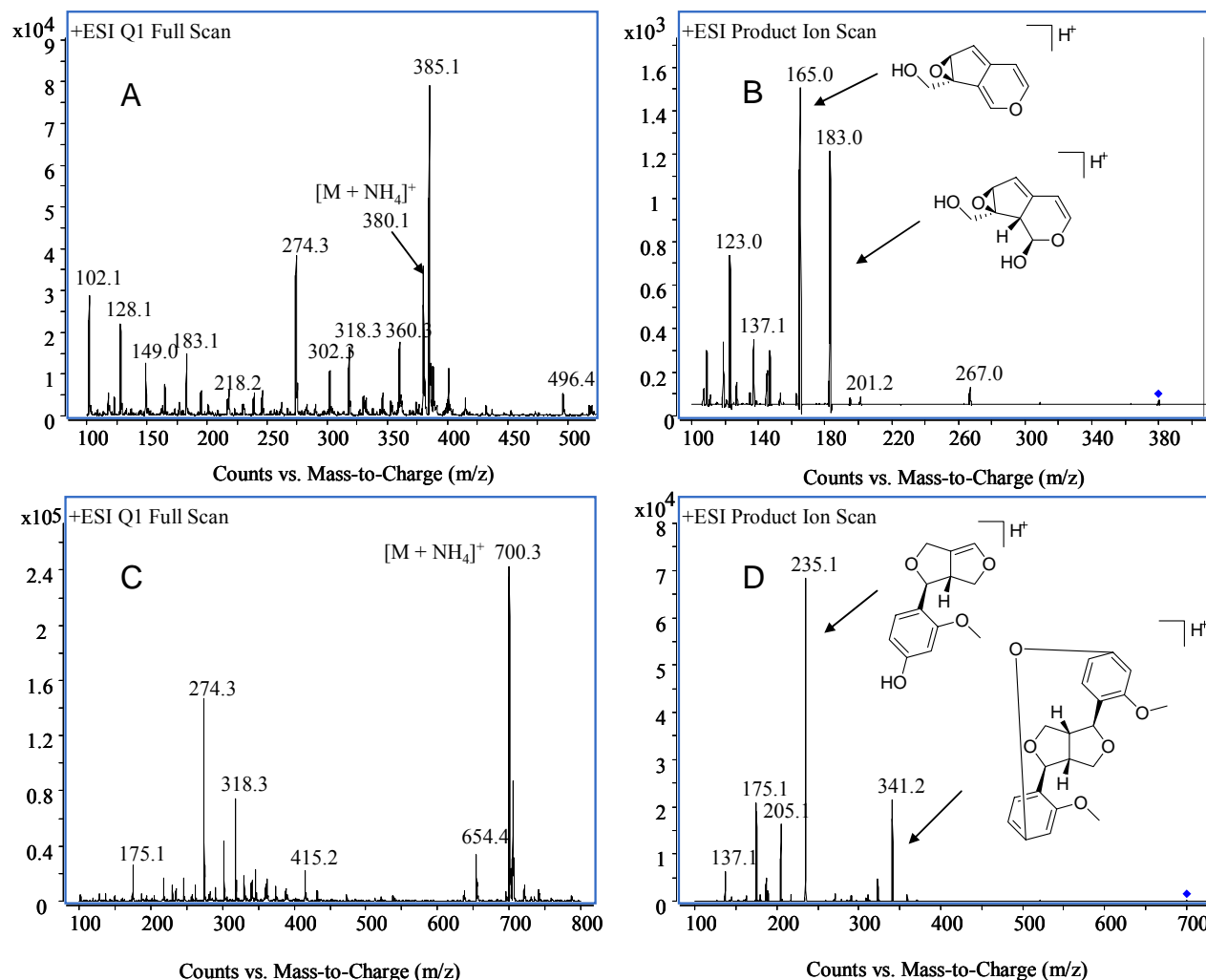


Fig. 2: Parent ion and product ion scan mass spectra of catalol (A, B), and pinoresinol diglucoside (C, D)

QC samples on three consecutive days. The precision expressed as the relative standard deviation (RSD) and the accuracy as the relative error (RE) should meet the acceptance criteria (within 15%).

Linearity and lower limit of quantitation (LLOQ)

Linearity was assessed by analyzing the eight-point calibration curves in plasma, in which each point in duplicate was analyzed in three consecutive days. The final concentrations of calibration standards were 1.00, 3.00, 10.0, 30.0, 100, 300, 1000 and 3000ng/mL. The standard curve was fitted by weighted linear regression ($1/x^2$) of analyte-internal standard peak area ratios.

The lower limit of quantification (LLOQ) was defined as the lowest concentration of standard at which the precision and accuracy were $\leq 20\%$.

Matrix effect

The matrix effect was evaluated by comparison of the peak area of analytes dissolved in the blank plasma

sample's precipitated solution with that of the analytes dissolved in the mobile phase. Three QC levels of PD (3.00, 100 and 2700ng/mL) were measured by analyzing three samples at each level. The blank plasma used in this study was from six different batches of the blank rat plasma. If the peak area ratio is less than 85% or more than 115%, a matrix effect is implied.

Extraction recovery

The extraction recovery of PD in plasma at three QC levels was investigated by comparing the peak areas of PD extracted from plasma samples with those of dissolved in the mobile phase.

Stability

The stability of PD in plasma was evaluated in triplicates of QC samples under different storage conditions: short-term stability (room temperature for 4h), long-term stability (-20°C for 18 days), three freeze-thaw cycles ($-20^\circ\text{C} \leftrightarrow 20^\circ\text{C}$), and the post-preparative stability (in autosampler tray at 20°C for 12 h).

Pharmacokinetic application

The study was approved by the Institutional Animal Ethics Committee. The method was applied to the pharmacokinetic study of PD in rats. Twelve rats were divided into two groups at random. After fasting for 12h, rats were administered at a dose of 85mg/kg PD by gavage or 10mg/kg by intravenous injection via the tail vein. The drug was formulated by dissolving PD with physiological saline to obtain a concentration of 1.0mg/mL for intravenous administration. 127.5mg of PD powder was dissolved in 15mL pure water to get 8.5mg/mL solution for oral administration. The rats were fasted for 2 h with free access to water after dosing. Blood samples (approximately 200 μ L) were collected from the vein of the eye ground into heparinized tubes at 0 (pre-dose), 3, 5, 10, 15, 30, 60, 90, 120, 180 and 300 min after administration. After the blood was centrifuged at 3000 rpm for 10 min, the separated plasma was immediately transferred into 1.5mL neat centrifuge tube and then stored at -20°C until LC-MS/MS analysis.

STATISTICAL ANALYSIS

To obtain the pharmacokinetic parameters of PD, the concentration-time data expressed as mean \pm SD were calculated by a noncompartmental model from DAS 2.0 Software (China State Food and Drug Administration). Bioavailability was calculated according to the following equation:

$$\text{bioavailability (F\%)} = \frac{\text{AUC}_{0-\infty}(\text{po}) \times \text{dose (iv)}}{\text{AUC}_{0-\infty}(\text{iv}) \times \text{dose (po)}} \times 100.$$

RESULTS

MS/MS optimization

MS parameters were optimized by infusing the standard solution (100 μ g/mL) into the mass spectrometer in the SRM mode. The signal intensity obtained in positive mode was much higher than that in negative ion mode under the respective positive/negative scan mode. The PD-related ammonium ion ($[\text{M} + \text{NH}_4]^+$, m/z 700.3) was chosen as the precursor ion in the Q1 spectrum and further was used to obtain product ion spectra in the Q3 spectrum. Similarly, the IS-related ammonium ion ($[\text{M} + \text{NH}_4]^+$, m/z 380.1) was used to get the product ions in MS/MS scan mode (fig. 2). The most sensitive mass transition was monitored from m/z 700.3 to 235.1 for PD, and from m/z 380.1 to 165.0 for IS.

Method validation

Selectivity

No interference peak was observed at their retention times of PD and IS. Fig. 3 shows typical chromatograms for a blank rat plasma (fig. 3A); a blank rat plasma spiked with PD (1.00ng/mL) and IS (5000ng/mL; fig. 3B); a rat plasma sample obtained at 90min after oral administration

of 85mg/kg PD (fig. 3C) and at 90 min after iv injection of 10mg/kg PD (fig. 3D).

Accuracy and precision

The intra- and inter-day accuracies for PD were -3.7%~6.5% and -3.9%~7.3%, respectively (table 1). The intra- and inter-day precisions for PD were 5.6%~8.9% and 0.5%~2.0%, respectively.

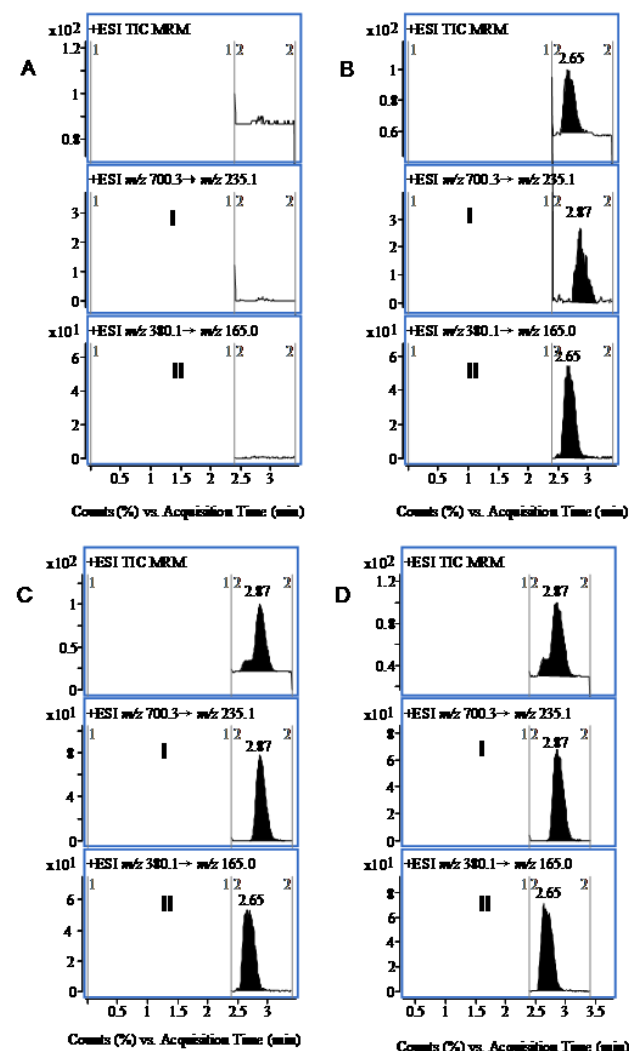


Fig. 3: Chromatograms of pinoresinol diglucoside and IS by MRM scan mode: (A) blank plasma sample; (B) LLOQ of pinoresinol diglucoside (1.00ng/mL) or IS (5000ng/mL); (C) plasma sample 90min after intravenous administration of pinoresinol diglucoside (10 mg/kg); (D) plasma sample 90min after oral administration of pinoresinol diglucoside (85mg/kg).

Linearity and LLOQ

The standard calibration curves for PD was assessed over the ranges of 1.00-3000ng/mL with the value of LLOQ (1.00ng/mL). A typical regression equation for the calibration curves was $y = 6.20 \times 10^{-3} x + 2.46 \times 10^{-2}$, $r^2 =$

0.9962, where y is the peak area ratio of PD to IS, and x represents the concentration of PD in rat plasma.

Matrix effects and extraction recovery

The matrix effect ranged $95.4 \pm 1.7\%$, $93.2 \pm 7.1\%$, $95.8 \pm 3.8\%$ for PD at low, medium and high QC level, and $95.8 \pm 1.6\%$ for IS, respectively. The data indicated no significant matrix effect resulted from co-eluting endogenous substances with the ionization of PD or IS under the described LC-MS/MS condition. The extraction recoveries of PD from rat plasma were $104.9 \pm 2.7\%$, $97.9 \pm 4.7\%$ and $102.7 \pm 6.4\%$ for the QC samples at three concentration levels, respectively, whereas $105.8 \pm 4.4\%$ for IS at 5000ng/mL. The above results were shown to be consistent and reproducible.

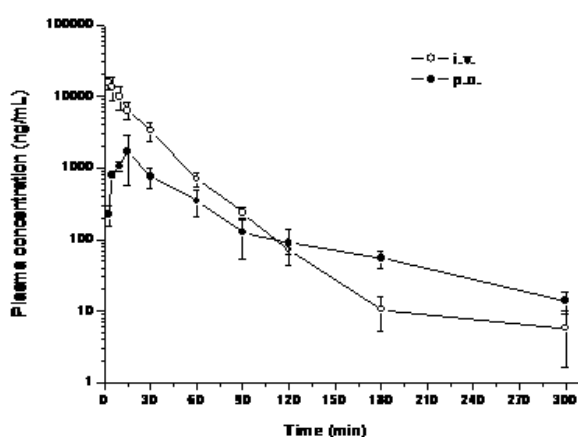


Fig. 4: Mean plasma concentration-time profile of pinoresinol diglucoside in rats after intravenous administration of 10mg/kg (A), and oral administration of 85mg/kg (B).

Stability

Stability data of PD were indicated in table 2, which demonstrated a good stability of PD in plasma stored at -20°C for 18 days, during three freeze-thaw cycles ($-20^{\circ}\text{C} \leftrightarrow 20^{\circ}\text{C}$), at ambient temperature for 4h, and in autosampler tray at 20°C for 12h.

Dilution integrity

The dilution integrity precision of the QC samples was $\leq 4.1\%$ and the accuracy was within $\pm 8.8\%$. Dilution integrity could cover the dilution for applying to the prepared samples.

Pharmacokinetic study

The LC-MS/MS method described above was successfully applied for evaluating the Pharmacokinetics in which the concentrations of PD in rat plasma were determined for 5.0h after intravenous injection (10mg/kg, $n=6$) and oral administration (85mg/kg, $n=6$), respectively. The Pharmacokinetic profiles of PD are presented in fig. 4 and the main parameters are indicated in table 3.

DISCUSSION

The mobile phase was considered as a key factor for influencing the chromatographic separation and ionization efficiency of the analytes. In the present experiment, the optimized mobile phase consisted of methanol-10 mM ammonium acetate buffer-acetic acid (50:50:0.15, v/v/v), which proved that little matrix interference and good recovery was observed as well as proper retention time (PD, 2.87 min; IS, 2.65 min).

Extraction of the drug from plasma was difficult as PD has high polarity with two glucose units, so simple protein precipitation with methanol was used to obtain excellent reproducibility with three QC concentration levels.

The absolute bioavailability of PD in rats was low with a value being only 2.5%, which was calculated according to the $\text{AUC}_{0-\infty}$ value obtained after oral and iv administration. The result suggested that PD was poorly absorbed *via* the gastrointestinal segment, which might result from the intestinal flora-induced degradation and intestinal absorption difficulty due to large molecular weight of the drug, as well as efficient metabolism of the drug in intestine following oral administration to rats (Song *et al.*, 2013; Zhao *et al.*, 2014). However, this reason of poor absorption needs further investigation. The information described above might be helpful for further studies on the Pharmacokinetics of PD, and beneficial for the application of this drug in clinical therapy.

CONCLUSION

A selective, sensitive, and rapid LC-MS/MS method for the determination of PD in rat plasma has been developed and validated as per the FDA guideline. The results showed that the absorption of the drug could be significantly improved by intravenous injection compared

Table 1: Precision and accuracy of pinoresinol diglucoside in rat plasma (intra-day, $n = 6$; inter-day, $n = 18$, 3 days).

Spiked concentration (ng/mL)	Intra-day ($n = 6$)			Inter-day ($n = 18$)		
	Measured Concentration (ng/mL)	Accuracy (%RE)	Precision (%RSD)	Measured concentration (ng/mL)	Accuracy (%RE)	Precision (%RSD)
3.00	2.89 ± 0.18	-3.7	5.6	2.88 ± 0.15	-3.9	0.5
100	100.38 ± 4.97	0.4	5.8	100.62 ± 5.50	0.6	0.9
2700	2876.06 ± 53.33	6.5	8.9	2896.51 ± 282.15	7.3	2.0

Table 2: Stability of pinoresinol diglucoside in rat plasma under different conditions ($n = 3$).

Conditions	Measured concentration (ng/mL)	Accuracy (%RE)	Precision (%RSD)
3.00ng/mL (Low)			
0 h	2.82±0.18	-6.1	6.4
Long-term stability (-20°C, 18 days)	3.06±0.12	2.2	4.0
Freeze-thaw cycles (-20°C ↔ 20 °C, three times)	2.85±0.06	-5.1	2.1
Ambient (20 °C, 4 h)	3.00±0.19	-0.1	6.3
Autosampler tray for (20°C, 12 h)	2.89±0.09	-3.6	2.9
100ng/mL (Medium)			
0 h	106.00±10.88	6.0	10.3
Long-term stability (-20°C, 18 days)	107.40±10.84	7.4	10.1
Freeze-thaw cycles (-20°C ↔ 20 °C, three times)	97.59±6.05	-2.4	6.2
Ambient (20 °C, 4 h)	95.96±6.91	-4.0	7.2
Autosampler tray for (20°C, 12 h)	105.90±11.03	5.9	10.4
2700ng/mL (High)			
0 h	2851.86±125.04	5.6	4.4
Long-term stability (-20°C, 18 days)	2867.79±62.75	6.2	2.2
Freeze-thaw cycles (-20°C ↔ 20 °C, three times)	2872.26±64.04	6.4	2.2
Ambient (20 °C, 4 h)	2768.78±191.98	2.5	6.9
Autosampler tray for (20°C, 12 h)	2883.34±129.12	6.8	4.5

Table 3: The main pharmacokinetic parameters of pinoresinol diglucoside after intravenous and oral administrations (mean ± SD, $n = 6$).

Parameters	Administration mode	
	Intravenous (10 mg/kg)	Oral (85 mg/kg)
T_{max} (h)	—	0.292±0.102
C_{max} (ng/mL)	13497±4752	17621±1123
$t_{1/2}$ (h)	0.325±0.025	1.18±0.17
MRT (h)	0.332±0.014	0.846±0.194
CL or CL/F (L/h*kg)	1.98±0.60	3.51±0.67
V_d (L/kg)	0.912±0.228	2.67±0.55
AUC _{0-t} (ng*h/mL)	5396±1217	1144±218
AUC _{0-∞} (ng*h/mL)	5485±1666	1167±220
F (%)	—	2.5

with oral administration, and suggested that the intravenous route might be preferentially considered in the evaluation of preclinical Pharmacodynamics of the drug.

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