

Pharmacokinetics, intestinal absorption and microbial metabolism of single platycodin D in comparison to *Platycodi radix* extract

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ABSTRACT

Background: *Platycodi radix*, the dried root of *Platycodon grandiflorum* A. DC, has been widely used as food and herb medicine for treating cough, cold and other respiratory ailments, and platycodin D (PD) is one of the most important compounds in *Platycodi Radix*. **Objective:** The purpose of this study was to compare the pharmacokinetic characteristics, intestinal absorption and microbial metabolism of PD in monomer with that in *Platycodi radix* extract (PRE). **Materials and Methods:** In the pharmacokinetic study, the concentrations of PD in rat plasma were determined by ultra-performance liquid chromatography-tandem mass spectrometry and the main pharmacokinetic parameters were calculated by data analysis software (DAS). Besides, *in vitro* Caco-2 cells and fecal lysate were performed to investigate the intestinal absorption and metabolism, respectively. **Results:** The results from pharmacokinetics showed that the area under the curve, the peak concentration the time to reach peak concentration and mean residence time of PD in PRE were enhanced significantly compared with that in single PD. Caco-2 cells transport study indicated that the absorption of PD both in monomer and in PRE were poor owning that the permeability of PD were $< 1/10^6$ cm/s. The hydrolysis degree of PD in PRE was significantly lower than that in monomer PD in fecal lysate, which might be illustrated by the other ingredients in PRE influenced the hydrolysis of PD via gut microbiota. **Conclusion:** These findings indicated that the difference of microbial metabolism, not apparent absorption in intestine for PD between in monomer and in PRE contributed to their pharmacokinetic difference.

Key words: Intestinal absorption, microbial metabolism, pharmacokinetics, *Platycodi radix*, platycodin D

INTRODUCTION

Platycodi radix, the dried root of *Platycodon grandiflorum* A. DC, has been widely used as food and herb medicine for treatment of cough, cold, sore throat, tonsillitis, bronchitis, bronchial asthma, and other respiratory ailments.^[1,2] Till now, more than 40 triterpenoid saponins, the major active components of *P. radix*, have been reported.^[3-5] In these saponins, platycodin D (PD) shown in Figure 1 have a high content in this crude drug, which applied as a chemical marker for the quality control of *P. radix* in the Chinese

pharmacopeia (2010 edition).^[1] The compound displays many beneficial properties,^[6] such as anti-inflammatory,^[7,8] hyperlipidemic,^[9,10] anti-allergic properties,^[11] as well as protective effects on oxidative hepatotoxicity,^[12] stimulating apoptosis in cancer cells and exerting anticancer activities.^[13-15]

It was reported that the oral absolute bio-availability of PD in monomer was significantly lower than that in *Platycodi radix* extract (PRE).^[16-18] Thus, the pharmacokinetic profiles of single PD could not represent that in PRE, which might be demonstrated by the absorption or metabolism difference result from other components.

In this study, a comparative pharmacokinetic study was performed to investigate the differences of pharmacokinetic behaviors in rats after oral administration of PRE and single PD. Besides, Caco-2 cells and fecal lysate models were

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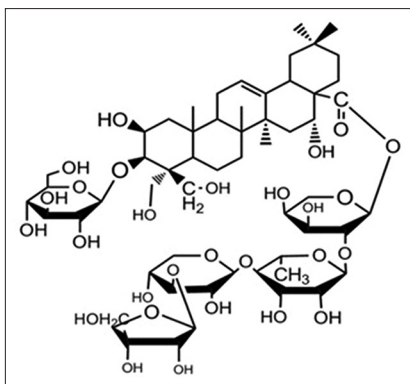


Figure 1: Chemical structures of platycodin D

performed to investigate the transmembrane behavior and intestinal microbial metabolism of PD both in monomer and in PRE, respectively.

MATERIALS AND METHODS

Materials

The herb of *P. radix* was purchased from Nanjing Yifeng Pharmacy (Nanjing, China). Reference substances of PD and hesperidins (internal standard [IS]) were purchased from Sichuan Weikeqi Biological Technology Co., Ltd. (Chengdu, China). Both of the purities were above 98% by high-performance liquid chromatography (HPLC) analysis. Heated inactivity fetal bovine serum (FBS), trypsin-ethylenediaminetetraacetic acid (EDTA), Dulbecco's Modified Eagle's Medium (DMEM), nonessential amino acid solution, L-glutamine, penicillin-streptomycin, were purchased from Gibco Laboratory (Invitrogen Co, Grand Island, NY, USA). HPLC-grade acetonitrile was supplied by Merck (Darmstadt, Germany), and formic acid with a purity of 99% was of HPLC grade (ROE, USA). Deionized water (18 M Ω) was prepared by a Milli-Q system (Millipore, Milford, MA, USA). Other reagents and chemicals were of analytical grade.

Preparation of *Platycodi radix* extract

The herb of *P. radix* (50 g) was immersed in 500 mL deionized water for 0.5 h and then decocted to boil for 2 h. The extracted solution was filtered through two layers of gauze. Ethanol was added to the filtrate to precipitate the polysaccharide and protein. This sample was kept for 24 h at 4°C and then filtered through the filter paper and the filtrate was evaporated to obtain PRE, a final concentration of 1.0 g/mL (equivalent to dry weight of raw materials). The content of PD in PRE was quantitatively determined at 1.9 mg/mL by ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS).

Animals and oral pharmacokinetic study

Male Sprague-Dawley rats (weighing 250 \pm 20 g) were obtained from the Lab Animal Center of Nanjing University of Chinese Medicine and housed with free access to food and water. The experimental protocol was approved by the University Ethics Committee for the use of experimental animals and all animal studies were carried out according to the guide for care and use of laboratory animals. The animals were acclimatized to the facilities for 3 days, and then fasted for 12 h before each experiment.

Platycodi radix was used orally in clinics, and the typical dosage is 30–60 g/person (60 kg of body weight).^[16] If the dosage in a rat was reasonably calculated by converting the human dosage with the conversion rate at 1:6.3 (human to rat), thus, the oral dosage in a rat PK study of PRE may be designed to be 6 g/kg. Meanwhile, the oral dosages of PRE in the previous studies and quantification sensitivity of instruments were all taken into consideration in our present study. Eventually, the oral dosage of PRE and PD was set at 10 g/kg and 20 mg/kg (corresponding equivalent PD), respectively.

The rats were randomized into two groups, six in each group: Rodents in group A were administered an oral dose of 20 mg/kg PD; rodents in group B were administered an oral dose of PRE (10 g/kg, corresponding equivalent PD). About 300 μ L blood samples were collected into heparinized tubes via the postorbital venous plexus veins before drug administration and at 0.083, 0.167, 0.333, 0.667, 1, 2, 3, 4, 6, 8, 12 and 24 h after drug administration. Then the blood samples were immediately centrifuged at 5,000 rpm for 10 min and aliquots (100 μ L) of plasma were spiked with 10 μ L IS solution (92 ng/mL) by vortexing for 30 s, then 300 μ L of methanol was added and the mixture was vortexed for 5 min. The well vortexed solutions were then centrifuged at 17,000 rpm for 10 min and separate the precipitated protein, 5 μ L of the supernatant were injected into the UHPLC-MS/MS system for analysis.

To obtain the pharmacokinetic parameters of PD, the concentration time data were processed by noncompartmental methods using data analysis software (DAS, Version 3.0, Chinese Pharmacological Society, China). The parameter the time to reach peak concentration and C_{max} (the peak concentration) of PD were obtained directly from the time point corresponding to peak concentration and peak concentration in rat plasma, respectively. Pharmacokinetic parameters including half-life, area under the curve (AUC), mean residence time (MRT) were determined by DAS software.

Cell culture

Caco-2 cell line was obtained from American Type Culture Collection, (Rockville, MD, USA). Cells were grown routinely

on 75 cm² plastic culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA) in DMEM containing 25 mM D-glucose, 25 mM HEPES, 44 mM NaHCO₃, supplemented with 10% (v/v) FBS, 1% (v/v) nonessential amino acid solution, 1% (v/v) L-glutamine, 1% (v/v) penicillin-streptomycin at 37°C in an atmosphere of 5% CO₂ and 90% relative humidity. The medium was replaced every 2–3 days after incubation. Cells were passaged, 1:5, approximately every 5 days (at 70–80% confluence) using 0.25% trypsin and 0.02% EDTA. For the transport experiments, cells were seeded at a density of 5 × 10⁴ cells/cm² onto permeable polycarbonate insert (0.6 cm², 0.45 μm pore size, Millipore, USA) in 24-well tissue culture plates (NUNC, Roskilde, Denmark). Media in the culture plates was changed every 2 days for the 1st week following seeding then replaced every day. Caco-2 monolayers were used for the experiments between days 21 and 24 postseeding. Cell passages between 32 and 40 were used in the experiment. The qualities of the monolayers grown on the permeable membrane were assessed by the transepithelial electrical resistance (TEER) of the monolayers at 37°C using a Millicell-ERS apparatus (Millipore, Bedford, MA, USA). Only monolayers displaying TEER values above 300 Ωcm² were used in the experiments, which indicated that the cell monolayers were not leaky.

Transcellular transporter of platycodin D across Caco-2 cell monolayer

The transport study across Caco-2 monolayer was investigated by a modified version of a previously published method.^[19] Briefly, 0.4 mL of PD solution (0.45 μg/mL) or PRE solution (corresponding equivalent PD) in hanks's balanced salt solution (HBSS) was loaded on apical side of the cell monolayer and 0.6 mL of blank HBSS onto the basolateral side. Aliquots of 0.1 mL and 0.15 mL of samples were taken sequentially at time points 0, 30, 60, 90, 120 min from donor and receiver side of the cell monolayer respectively. Drug solution and HBSS media were added to apical or basolateral side immediately to compensate for sampling volume lost. The pH of HBSS in both donor and receiver side was 7.4. A volume of 10 μL of an IS (92 ng/mL hesperidins in acetonitrile) was added to 100 μL samples right after sampling. Caco-2 samples were blown dried by nitrogen and reconstituted with 20 μL methanol for UHPLC-MS/MS.

The apparent unidirectional permeability, from apical to basolateral side (P_{app}), was calculated according to the following equation

$$P_{app} = \frac{dQ/dt}{A \times C_0}$$

where the dQ/dt (μg/min) is the drug permeation rate, A is the cross sectional area (0.6 cm²), and C_0 (μg/mL) is

the initial PD concentration in the donor compartment at $t = 0$ min.

Fecal lysate preparation

Fecal lysate was prepared by the method described previously with some modifications.^[20] Briefly, Fresh feces of ten rats were collected, and about 1 g were immediately mixed with 9 mL ice-cold 0.1 mM phosphate buffer solution (PBS), (pH 7.4) and vortexed for 5 s followed by centrifugation at 1,000 rpm and 4°C for 15 min. The pellet was resuspended in 10 mL ice cold PBS, then sonicated in ice water bath for 45 min, and finally centrifuged at 15,000 rpm and 4°C for 30 min. The supernatant was aliquoted and stored at -70°C. Protein concentrations were detected by the bicinchoninic acid protein assay kit with bovine serum albumin as the standard.

Hydrolysis of platycodin D in fecal lysate

Thawed fecal lysate (100 μL) was transferred to disposable glass vials, then 10 μL of PD solution (1.1 μg/mL) or PRE solution (0.5 mg/mL, corresponding equivalent PD) and 90 μL PBS solution were added to the samples. The mixture was incubated at 37°C and 120 rpm in an vapour-bathing constant temperature vibrator (Asheville, NC) for 48 h. Samples (100 μL) were collected at 0, 0.5, 1, 2, 4, 8, 12, 24, 36 and 48 h, then 10 μL of 92 ng/mL hesperidins (IS) in 100% methanol was immediately added to the collected sample to stop the reaction. The samples were vortexed for 3 min and centrifuged at 17,000 rpm for 10 min. The supernatant was injected into the UHPLC/MS-MS to for analysis of PD. Every given time experiments were performed independently in triplicate.

Quantitation of platycodin D in biological matrices

The UHPLC/MS-MS was an ultimate 3000 series UHPLC system (Dionex, Genmany) consisting of a quaternary pump, an autosampler, a vacuum degasser, and thermostatically controlled column apartment equipped with thermo scientific quantum vantage™ mass spectrometer system (Thermo-Fisher Scientific, Bremen, Germany), including a hybrid triple quadrupole linear ion trap mass spectrometer coupled with an electrospray Ionization (ESI) source. All data were analyzed by Xcalibur Software (Thermo Fisher Scientific, SanJose, USA) from Thermo-Fisher Scientific. PD and IS were monitored in the negative mode (ESI). High-purity nitrogen was used as sheath gas (45 arb) and auxiliary gas (25 arb) respectively. High-purity argon was used as the collision gas (1.5 mTorr). spray voltage, 3.0 kV; capillary temperature, 350°C; vaporizer temperature, 450°C. Q1 and Q3 quadrupoles were set at unit resolution. The quantification was performed by selected reaction monitoring of the transitions of m/z 1223.5 → 681.5 for PD, and m/z

609.2 → 301.1 for hesperidins (IS) respectively. The chromatographic analysis was achieved on a hypersil gold column (100 mm × 2.1 mm i.d., 1.9 μm particle size; Thermo, USA). The mobile phase consisted of 0.05% formic acid in water (A) and acetonitrile (B) at a flow rate of 0.4 mL/min. The UHPLC gradient system began with 15% B at 0–1 min, 15–60% B at 1–5 min, 60–100% B at 5–6 min. The column temperature was maintained at 40°C, while the sample-tray temperature was kept at 4°C.

The method had linear calibration curves over the concentrations of 1.95–1500.0 ng/mL for PD in rat plasma, 1.47–1500 ng/mL in HBSS buffer and 1.47–1500 ng/mL in inactivated fecal lysate solution. The extraction recoveries were 74.3–86.6%, 101.3–104.8% and 94.4–97.5% for PD in rat plasma, HBSS buffer and inactivated fecal lysate solution respectively. The lower limit of quantification for PD was 1.95 ng/mL in rat plasma, 1.47 ng/mL in HBSS buffer and 1.47 ng/mL in inactivated fecal lysate solution, respectively. The intra and inter-batch precision and accuracy were <20% for all quality control samples in rat plasma, transport buffer and fecal lysate solution.

Data analysis

The data in this paper were presented as mean test for each group, if not specified otherwise. Significance was assessed by one way analysis of variance and Student's *t*-test. A *P* value of < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Oral pharmacokinetics of platycodin D in rats

The plasma concentrations of PD in rats were determined after following oral administration of single PD at 20 mg/kg and equivalent dose of PRE. The results [Figure 2 and Table 1] showed that single PD was rapidly

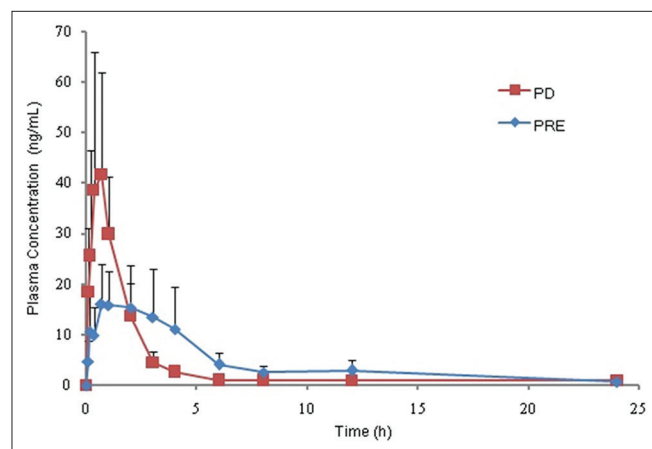


Figure 2: Mean concentration-time profiles of platycodin D in rat plasma after oral administration of *Platycodi radix* extract (10 g/kg) and single platycodin D (20 mg/kg) (mean ± standard deviation, *n* = 6)

absorbed into the circulatory system and reached its peak concentration. PD was detected in rat plasma at 10 min after oral administration of single PD and PRE, and reached C_{max} at approximately 30 min and 75 min with C_{max} approximately 44.45 ng/mL and 17.94 ng/mL, respectively. The $AUC_{(0-\infty)}$ of PD in rats receiving the single PD was low (73.00 ± 24.17 ng h/mL), which was consistent with the previous results.^[16,18] The $AUC_{(0-\infty)}$ of PD after an equivalent dose of PRE to rats was observed to 96.06 ± 48.51 ng h/mL, which was significantly higher than that in rats receiving the single PD, indicating PD in PRE is more exposed to blood circulation than single PD. MRT describes the average time for all the drug molecules to reside in the body. $MRT_{(0-t)}$ of PD was also increased from 1.38 ± 0.20 h (single PD) to 6.10 ± 1.03 (PD in PRE), which showed PD in PRE duration *in vivo* prolonged, compared to single PD. Previously, Zhan *et al.*,^[18] reported the pharmacokinetics of PD between in monomer PD and in PRE. There were some differences between the previous report and our study, which may be attributed to the different doses to rats. In Zhan's study, the oral dosage of single PD and PRE was 80mg/kg and 18.75 g/kg respectively, while the oral dosage was 20 mg/kg and 10 g/kg (corresponding equivalent PD) in our present study. The appearance indicated that the other ingredients contained in PRE, like saponins, may influence the absorption or metabolism of PD.

Transcellular transport of platycodin D across Caco-2 cell monolayer

We determined the apparent permeability of PD both in PRE and in monomer PD in Caco-2 cell monolayers, a model employed to mimic human intestinal absorption characteristics.^[21,22] Transport of 0.45 μg/mL PD and PRE (200 μg/mL, equivalent to 0.45 μg/mL PD) from apical side to basolateral side in Caco-2 cells were determined within 2 h to understand whether the coexisting components in PRE could influence the transport of PD.

The result of 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay showed that single

Table 1: Pharmacokinetic parameters of PD in rats following oral administration of PRE (10 g/kg) and single PD (20 mg/kg) (mean±SD, *n*=6)

Parameter	PD	PRE
C_{max} (ng/mL)	44.45±22.40	17.94±9.33*
$t_{1/2}$ (h)	1.32±0.64	2.86±1.07*
T_{max} (h)	0.44±0.17	1.22±0.62*
$AUC_{(0-t)}$ (ng h/mL)	71.26±24.17	88.97±45.42
$AUC_{(0-\infty)}$ (ng h/mL)	73.00±24.17	96.06±48.51*
$MRT_{(0-t)}$ (h)	1.38±0.20	6.10±1.03*

**P*<0.05 compared with PD group. PD: Platycodin D; PRE: Platycodi radix extract; AUC: Area under the curve; MRT: Mean residence time; C_{max} : The peak concentration; T_{max} : The time to reach peak concentration; SD: Standard deviation; $t_{1/2}$: Half-life

PD and PRE at the working concentrations had no toxicity on Caco-2 cells within 2 h. The TEER values ($>300 \Omega\text{cm}^2$) were not different significantly before and after the transport experiment. The results showed that the permeability of PD in monomer and in PRE was $3.52 \times 10^{-7} \text{ cm/s}$ and $2.11 \times 10^{-7} \text{ cm/s}$ respectively, corresponding to incomplete absorption in humans ($1 \times 10^{-6} \text{ cm/s}$),^[21] which indicated that PD, like a lot of saponins, was poorly permeable.^[23] PD in PRE also exhibited poor permeability but a little lower than that of in monomer PD, which implied that the coexisting components in PRE could affect the transport of PD. A lot of other platycodins in PRE may competitively inhibit the transport of PD from apical side to basolateral side. Transcellular transport across Caco-2 cell monolayer declared that the absorption did not result in the phenomenon that higher level of PD exposure in PRE than single PD.

Hydrolysis of platycodin D in fecal lysate

The result of *in vitro* hydrolysis study of PD by rat fecal lysate was presented in Figure 3. PD was hydrolyzed rapidly in fecal lysate, and decreased by 64% within 30 min. The intensity of hydrolyzation slightly diminished and the concentration of PD plateaued around 2 h, only about 20% PD presented at this time point. There are two unbranched sugar chains attached to the carbons C-3 and C-28 in the aglycones in PD, and each chain is composed of one (C-3) and four monosaccharide (C-28) residues. In intestinal bacteria, the major pathway of metabolism of PD is the hydrolysis of C-3 glycoside, followed by acetylation at the multi-site or, alternatively, further hydrolysis at the C-28 oligosaccharide and dehydroxylation of the aglycone part.^[24,25] Furthermore, we are identifying the intestinal bacteria, such as β -glucosidases, potentially responsible for this metabolism to facilitate future metabolic studies of PD.

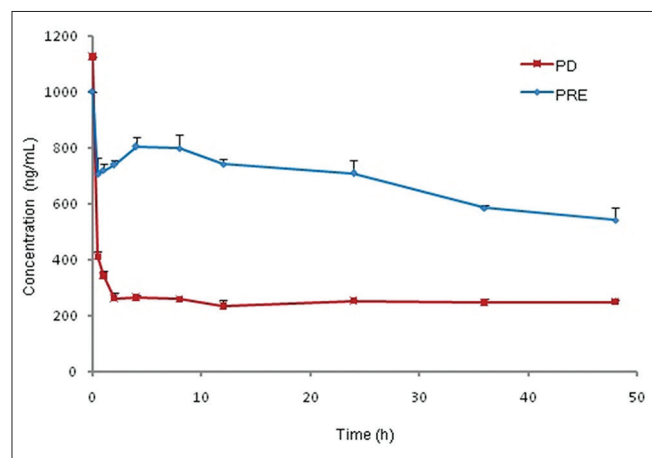


Figure 3: Metabolic profile of platycodin D from single platycodin D (1000 ng/mL) and *Platycodi radix* extract solution (0.5 mg/mL, corresponding equivalent platycodin D) in rat fecal lysate (mean \pm standard deviation, $n = 3$)

Be similar to single PD, PD in PRE was hydrolyzed immediately in a remarkably short time (30 min), about 30% PD disappeared. However, the concentration of PD increased slightly within 1–4 h, and then the hydrolyzation eased step by step. The residue of PD in PRE was more about 1.6 fold than free PD in fecal lysate within 48 h. This pattern of distinctive hydrolysis in fecal lysate between single PD and PD in PRE, to a large extent accounted for their difference of pharmacokinetics. In PRE, there are many platycodins, like PD, such as PD 2, PD 3, and platycoside E. All these platycosides can be defined as bidesmosidic oleanane-type triterpenes with two sugar moieties: A glucose unit attached through an ether linkage at C-3 of a triterpene, and the other embracing arabinose, rhamnose, and xylose in sequence with attachment of a glycoside linkage between C-28 and arabinose.^[5] These bidesmosidic saponins can be easily transformed into second platycodins by alkaline hydrolysis or intestinal microflora. So the other platycodins of PRE may be deglycosylated to PD under the microbial hydrolysis, which accounted for the concentration of PD in PRE increased slightly within 1–4 h. The biotransformation and metabolic profile of plentiful platycodins by intestinal microflora using high resolution mass spectrometry will be further investigated.

CONCLUSION

In summary, the pharmacokinetics, intestinal absorption and microbial metabolism of PD both in monomer and in PRE have been studied. The transmembrane behavior of PD was investigated by Caco-2 cells, showed it could not be absorbed well in intestine. It was demonstrated that the distinctive hydrolysis, not absorption, was the main reason for their difference of pharmacokinetics. Better understanding of the interactions between PD and the coexisting platycodins in the PRE will be investigated for further development of *Platycodi Radix* resources.

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