Development of a UPLC-ESI-MS/MS Assay for 20(*S*)-Protopanaxadiol and Pharmacokinetic Application of Its Two Formulations in Rats

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An ultra-performance liquid chromatography–electrospray tandem mass spectrometry (UPLC-ESI-MS/MS) method was developed to investigate 20(*S*)-protopanaxadiol (PPD) pharmacokinetics in rats. Rat plasma samples were treated using a solid-phase extraction with satisfactory recovery (>81%). The method showed an excellent sensitivity that the limit of detection (LOD) and the lower limit of quantitation (LLOQ) of PPD were 0.5 and 2 ng/mL, respectively. The method was applied to the evaluation of pharmacokinetics from two types of PPD formulations. The PPD emulsion showed more rapid and efficient drug absorption, and higher and more persistent plateau concentration of PPD in plasma than PPD oil solution. PPD emulsion was demonstrated to be a promising dosage form. In spite of lower plateau plasma drug concentration, PPD oil solution was characterized by the easiness in preparation and the persistent, durative plateau plasma concentration of PPD, there is room to further improve its bioavailability.

Introduction

Ginseng has been widely used in Traditional Chinese Medicine for its beneficial effect; 20(*S*)-protopanaxadiol (PPD, dammar-24-ene-3β,12β,20*S*-triol) is the common aglycone of ginsenoside Rb$_1$, Rb$_2$, Rb$_3$, Rc, Rd, Rg$_3$ and Rh$_2$, which are regarded as the major bioactive components isolated from ginseng. It is also the metabolite of some ginsenosides[1-4] and the product of their acid or alkaline hydrolysis.[5,6] Previous research done by our collaborators demonstrated that PPD was the actual effective motif of many PPD-type ginsenosides and can exert the therapeutic action after administration (data not reported). Therefore, PPD, instead of the corresponding ginsenosides, is selected for the actual use. Previous studies have indicated that PPD showed powerful pleiotropic anti-cancer effects in several cancer cell lines and the capability of inhibiting metastasis,[7-9] and it thus was a potential therapeutic agent in the prevention and treatment of cancer.[7,10-13]

However, the poor solubility (about 3 μg/mL) limits the clinic use of PPD. So far, only two papers have reported PPD concentration in plasma from two PPD dosage forms (PPD capsule and hydroxypropyl-β-cyclodextrin inclusion).[14,15] The results showed low plasma concentration of PPD from those forms. Although the mixture of DMSO, Tween and saline solution can enhance the solubility and bioavailability of PPD, the potential side effects and unpredictable risk of the solvent prevented its application in clinics. Therefore, some new dosage form must be designed to resolve this problem. In this paper, PPD emulsion was prepared, and this is a dosage widely used to enhance the solubility of many hydrophobic drugs. For more practicality in industry production, PPD oil solution spiked with phosphatidylcholine was prepared as well to check its ability to enhance PPD absorption and possibly prolong the therapeutic effect. The aim of this study was to develop an ultra-performance liquid chromatography–electrospray tandem mass spectrometry (UPLC-ESI-MS/MS) assay for PPD and to compare the pharmacokinetic parameters and bioavailability of PPD in the two oral formulations after a single dose.

Experimental

Chemical, reagents and animals

20(*S*)-Protopanaxadiol (PPD) (purity > 99.9%) was provided by Shanghai Shengjia BioScientific Co., Ltd. (Shanghai, China). Finasteride (internal standard, IS) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Their chemical structures have been shown in Fig. 1. Soybean phosphatidylcholine (SbPC) was obtained from Zhengzhou Siwei Phospholipid Technology Co., Ltd. (Zhengzhou, China). Cholesterol (Chol) and Tween 80 were Sigma products. Methanol and acetonitrile were of HPLC grade (Merck, Darmstadt, Germany). Deionized water was prepared by a Milli-Q system (Millipore, Bedford, MA). All reagents and solvents were of analytical grade. All the products and materials used in this study comply with the
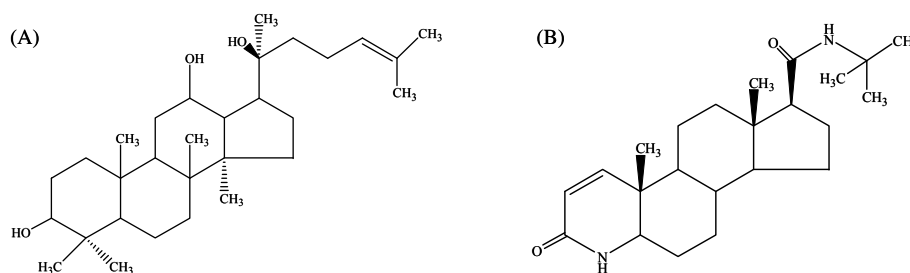
Fig. 1   Chemical structures of PPD (A) and the IS compound (B).

pharmaceutical and analytical standards, respectively. Sprague–Dawley (SD) rats were obtained from the Chinese Academy of Medical Sciences & Peking Union Medical College Institute of Animals (Beijing, China), and the rats were allowed free access to a commercial diet and water. The animal room was well ventilated and a regular 12-h light-dark cycle was maintained. All procedures adhered to the Guiding Principles in the Care and Use of Animals, and the protocol was approved by the local animal ethics committee.

Preparation of calibration standard and quality control (QC) samples

The stock solutions of PPD and IS at a concentration of 0.1 mg/mL were freshly prepared in MeOH:H_2O (2:1, v/v); 100 μL of plasma was added to 50 μL of the stock solution or diluted stock solution of PPD in the final concentration 2.5, 5, 10, 25, 75, 250, 750, 2000 and 5000 ng/mL of PPD in plasma, while low, medium and high PPD QC samples were prepared independently using a similar method at concentrations of 5, 500 and 2500 ng/mL. An IS working solution (100 ng/mL) was prepared by diluting the stock solution with MeOH:H_2O (2:1, v/v). All solutions were stored at –40°C when not in use.

Instrumentation and chromatographic conditions

The instrumentation system used in this work consisted of a Waters Acquity™ UPLC system equipped with a quaternary solvent delivery system, an autosampler fitted with a 10-μL loop and a QuattroMicro triple quadrupole mass spectrometer under multiple reaction monitoring (MRM) mode built by Waters (Milford, MA) and equipped with an electrospray ion source. Separation was carried out on an acquity BEH C18 column (50 × 2.1 mm, 1.7 μm, Waters, USA) with a C18 guard column (Security Guard, Waters, USA). The data were collected and processed using MassLynx 4.1 software. The isocratic HPLC mobile phase, consisting of a solution of 0.2% formic acid-acetonitrile (10:90, v/v) prepared daily and degassed before use, was pumped at a flow rate of 0.2 mL/min; the column temperature was set at 22°C. During the analyses, 5 μL of sample was injected by the autosampler. The optimized MS/MS parameters were selected as follows: The micromass Quattro triple quadrupole mass spectrometer was operated under the positive ion detection mode. The source temperature was set at 100°C, the desolvation temperature was 150°C. The optimized cone voltage values were 10 and 55 V for PPD and IS, respectively. Capillary voltage was set at 3.5 kV. The multiplier was set at 650 V and argon was used as the collision gas. Quantification was performed using MRM of the transitions m/z 461 → 444 for PPD and m/z 373 → 306 for the IS, with a dwell time of 0.7 s per transition. The optimized collision energy of 5 eV was used for the PPD and 30 eV was used for the IS.

Sample preparation

All frozen rat plasma samples (calibration standards, QC plasma samples and rat plasma samples) were thawed at room temperature, and 100 μL of plasma was added to 50 μL IS solution (100 ng/mL MeOH:H_2O) and 100 μL NaOH solution (0.1 M) in sequence. After being sonicated for 30 min, the mixture was centrifuged at 13000 rpm for 10 min. The supernatant was slowly added onto the solid-phase cartridge (HLB, 1 cc, Oasis, Waters, USA), which had been previously activated with 1 mL of methanol and balanced with 1 mL of water. After the sample had been absorbed by the cartridge, the cartridge was washed with 1 mL of 60% methanol in water and the PPD was then eluted with 1 mL of methanol, which was evaporated to dryness with nitrogen. The residue was redissolved in 250 μL methanol, and vortex mixed for 1 min. After centrifugation at 3000 rpm for 10 min, 5 μL of supernatant was injected into the UPLC system for analysis.

Method validation

Matrix effects for PPD and IS were investigated by comparing mean peak areas of post-extraction blank plasma samples spiked with PPD and IS with mean peak areas of post-extraction spiked with MeOH–H_2O at the same concentration.
Linearity of calibration curve and the lower limit of quantitation (LLOQ). The linearity of PPD over the range from 5 to 5000 ng/mL was assessed by linear regression of calibration curves based on peak area ratios of PPD to IS. Accuracy of the method was calculated by the equation: (determined concentration/ actual concentration) × 100% and the precision was expressed as the relative standard deviation (RSD). LOD was defined as the concentration with signal-to-noise ratio of three and LLOQ with signal-to-noise ratio of ten.
Recovery. The extraction recovery of PPD was determined by comparing the peak area of PPD in extracted QC samples using SPE with that of PPD reconstituted in blank plasma extracted using the same method at the corresponding concentrations.
Precision and accuracy. To evaluate intra-day and inter-days' accuracy and precision, we analyzed QC samples at concentrations of 5, 500 and 2500 ng/mL ($n = 6$) by assay of six replicates of each QC sample at different time points within the same day or over five consecutive days.
Stability. QC samples at concentrations of 5, 500 and 2500 ng/mL ($n = 6$) were used to investigate the stability of PPD in plasma under three storage conditions: (1) three freeze-thaw cycles of 24 h, (2) storage at –20°C for 60 days, (3) keeping at 4°C for 24 h, the same condition as that in autosampler. The stability of PPD in reconstitution solutions in the autosampler at room temperature for 6 h was also determined.

Pharmacokinetic application: study design and procedure
Preparation of PPD formulations. PPD solution of 2.5 mg/mL was prepared by dissolution of PPD in DMSO and then sequential addition of Tween 80 and physiological saline (DMSO:Tween 80:physiological saline 0.5:0.5:9, v/v/v).

PPD oil solution was prepared by dissolution of PPD in tea-seed oil, then addition of SbPC, Chol and 3% Tween 80, and then sonication at 75°C for 10 min.   The final ratio of the solution was tea-seed oil:SbPC:Chol (24:5:1, w/w/w), the final concentration of the PPD was 10 mg/mL and it would be diluted to 2.5 mg/mL before oral administration.

PPD emulsion was prepared by adding 3 volumetric times of sodium taurocholate solution (1 mg/mL) into the above PPD oil solution (containing 10 mg/mL PPD), followed by 5 min of vortex and 30 min of sonication at 60°C. The final concentration of the PPD in the emulsion was 2.5 mg/mL.

Pharmacokinetic study and statistical analyses. SD male 200 g rats were used to study the pharmacokinetics of PPD. Rats were fasted 12 h with free drinking water before the test. Eight rats in each group were administrated PPD oil solution, PPD emulsion by oral administration and PPD solution *via* a tail vein at a dose of 25 mg/kg body weight. Blood samples (0.3 mL) were drawn into heparin (2%) treated tubes at various predetermined time points (0.167, 0.5, 1.0, 2.0, 3.0, 5.0, 7.0, 9.0, 12 and 24 h) after the intravenous (i.v.) injection and (0.5, 1.0, 2.0, 3.0, 5.0, 7.0, 9.0, 12 and 24 h) after the lavage, and then centrifuged (3000 rpm, 10 min) to obtain clear plasma, the blood samples were frozen and stored at −80°C until analysis. Plasma PPD concentration *versus* time data for each rat was analyzed by non-compartmental methods with WinNonlin (Ver. 4.0, Pharsight Corp., USA): area under the drug concentration–time curve from zero to the last measurable PPD concentration sample time (AUC_{0-t}), maximum plasmatic drug concentration (C_{max}) and time to reach C_{max} (T_{max}), terminal rate constant (Kel), terminal half-life ($T_{1/2}$), the mean residence time (MRT), the volume of distribution (V) and total plasma clearance (Cl).

C_{max} and T_{max} were obtained directly from the concentration–time curve. AUC_{0-t} was calculated using the linear trapezoidal method. Kel was calculated by applying a log-linear regression analysis to at least the last three quantifiable concentrations of PPD.   Total plasma clearance (Cl) was calculated as the dose/AUC. The volume of distribution (V) was determined by Cl/Kel. $T_{1/2}$ was calculated as 0.693/Kel. Absolute bioavailability was determined by dividing the dose-normalized area under the concentration–time curve (AUC) resulting from oral administration by that resulting from intravenous administration, which was expressed as (AUC_{0-t}(oral) × Dose(i.v.))/(AUC_{0-t}(i.v.) × Dose(oral)) × 100%.

Results and Discussion

Choice of IS

When we judged by similarity of molecular structure, sensitivity, retention time and matrix interferences, finasteride and PD were found to be the best IS candidates for further evaluation. However, PD was not chosen to avoid possible interferences. As the consequence, finasteride was chosen as IS compound because of its shorter retention time and good separation from PPD, considering the price and availability of deuterated PPD.

Choice of the extraction method

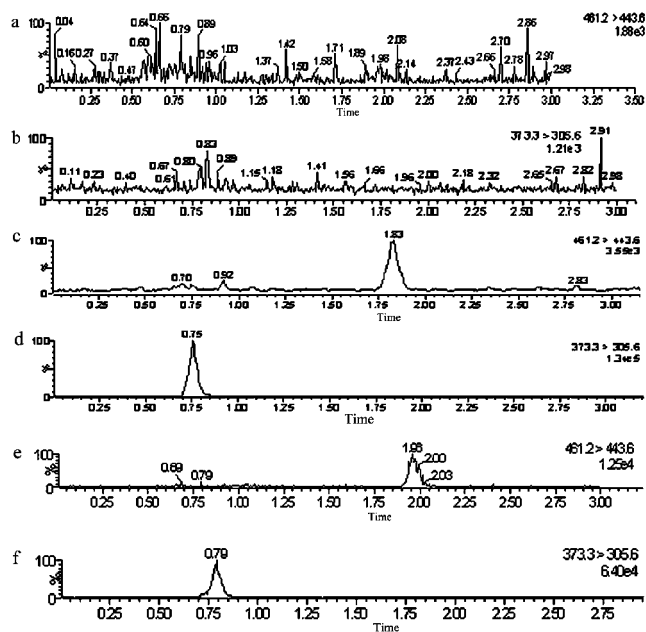The aim for sample preparation was to remove interferences

Fig. 2   Representative MRM chromatograms obtained from blank rat plasma monitored at m/z 461 → 444 (a) and at m/z 373 → 306 (b), from blank rat plasma spiked with PPD and IS monitored at m/z 461 → 444 (c) and at m/z 373 → 306 (d), and from a rat plasma sample 1 h after oral administration PPD, monitored at m/z 461 → 444 (e) and at m/z 373 → 306 (f).

from plasma samples with a suitable recovery using a minimum number of steps.   Several extraction procedures were investigated, including SPE and liquid–liquid extraction method. It was estimated that SPE could remove proteins and other interfering components in rat plasma with satisfactory drug recovery. The sample should be added slowly, and different concentrations of methanol were used for the final eluting procedure. The result indicated that the SPE column not only provided sufficient sample clean-up and gave higher recovery, but also gave better chromatograms with few interferences, and it was eluted with 60% methanol and methanol.

Bioanalytical method validation
Selectivity. The specificity of the method was investigated by comparing MS/MS chromatograms of PPD with those of blank plasma, sample spiked with PPD and a plasma sample from a rat at 1 h after oral administration of PPD.   In the MRM mode, blank rat plasma yielded clean chromatograms without significant interference to either PPD or IS, while peaks of PPD and IS were seen as sharp and steady in the plasma samples. The retention time for PPD was 1.83 min and it was well resolved from IS (0.75 min) (Fig. 2).
Linearity of calibration curve and LLOQ. A linear calibration curve with correlation coefficient (r) = 0.9981 was obtained in the plasma concentration range 5 – 5000 ng/mL as $y = 1.20976x + 36.1701$, where y indicates the peak area ratios of PPD to IS, and x indicates the plasma PPD concentration. LLOQ using 100 μL of plasma with acceptable accuracy and precision (<20%) was 2 ng/mL. A good signal-to-noise ratio ($S/N \geq 10$) was observed at the LLOQ, indicating that the corresponding value could be reached. The limit of detection (LOD) was estimated as the amount of PPD that gave a signal three times the noise ($S/N \geq 3$); it was calculated to be 0.5 ng/mL. Intra-assay precision and accuracy were suitable for

all the concentrations tested. RSD values were less than 10% at all concentrations.

Precision and accuracy. Inter- and intra-assay precision and accuracy were determined by repeated analysis of QC plasma samples (5500 and 2500 ng/mL) (n = 6) on the same day and on five consecutive days (Table 1). The RSD values for all samples of the intra-day and inter-days' accuracy and precision for PPD at three levels were less than ≤10.2%. The overall reproducibility of the method was acceptable.

Recovery. The relative recoveries of PPD were 81.06, 81.67 and 91.46% at 5, 500.0 and 2500 ng/mL in plasma, respectively, with RSD ≤15%.

Stability. The results of stability experiments at 5 and 2500 ng/mL in plasma indicated that PPD was stable under any of the storage conditions described above during sample preparation and in autosampler (RSD <10%), and that no

stability-related problems would be expected during the routine analysis of samples for the pharmacokinetic studies.

Pharmacokinetic study in rats. Figure 3 shows the mean plasma concentration–time curves in rats after oral administration of 25 mg/kg body weight of oil solution and emulsion of PPD. It is obvious that PPD emulsion maintained high plasma drug concentration over a much longer period of time, in contrast to PPD oil solution.

As seen from the shape of the plasma drug concentration–time curve, both PPD oil solution and PPD emulsion displayed as significant broad peaks (see Fig. 3). For PPD emulsion, the shape may be ascribed to the combined effect of drug release phase by the direct intestinal absorption into blood of PPD emulsion and indirect absorption of lymphatic passageway.

In case of PPD oil solution, as the result of very slow release of PPD from its oil solution for direct intestinal absorption, the prolonged absorption phase was mainly due to the lymphatic passageway of PPD absorption in the form of chyle. Because lymphatic circulation is usually one tenth of the velocity of blood flow, lymphatic absorption could play the role of extended release. And because of the presence of direct intestinal absorption of PPD emulsion, it was evident that the plateau plasma drug concentration of PPD emulsion was much higher than that of PPD oil solution.

As shown in Table 2, the C_{max} and AUC$_{(0-t)}$ values of PPD emulsion were higher than those of PPD oil solution. The result indicated that the bioavailability of PPD emulsion (22.37%) was better than that of PPD oil solution (20.74%).

After i.v. administration of PPD solution, PPD was absorbed into the blood through the form of molecules. The $T_{1/2}$ and Kel

Table 1   Intra-day and inter-day precision of the developed UPLC-MS/MS method for the determination of PPD

Nominal concentration/ ng mL^{-1}		Found concentration/ ng mL^{-1}	RSD, %
5	Intra-day	4.76 ± 4.10	9.8
	Inter-day	4.48 ± 4.23	10.2
500	Intra-day	497.8 ± 40.6	6.42
	Inter-day	493.5 ± 54.9	8.74
2500	Intra-day	2480 ± 298.7	9.91
	Inter-day	2513 ± 224.2	7.35

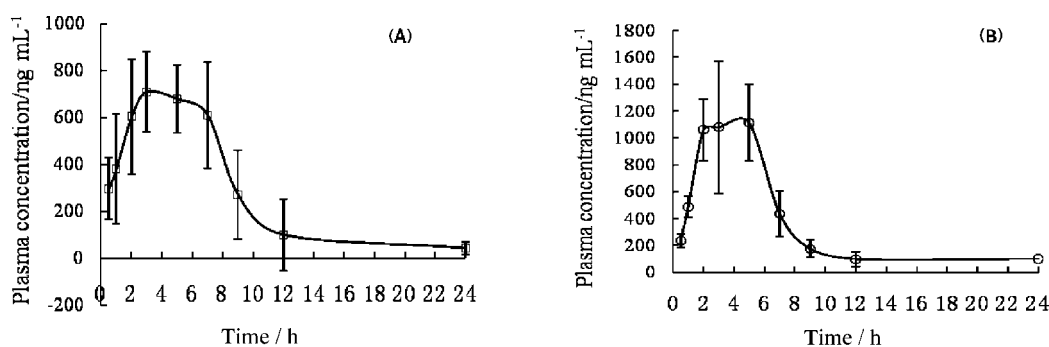Each value represents the mean ± SD (n = 6).

Fig. 3   Mean plasma PPD concentration *vs.* time curve after oral administration of oil solution (A) and emulsion of PPD (B) at a dose of 25 mg/kg body weight. Each point represents the mean ± standard error (n = 8).

Table 2   Summary of the pharmacokinetic parameters after oral administration of two PPD formulations and i.v. administration of PPD solution at a dose of 25 mg/kg body weight in rats

Parameter	Oil solution	Emulsion	PPD solution (i.v.)
C_{max} (ng/mL)	708.27 ± 136.61	1114.49 ±152.92	3850.76 ±15381.78
T_{max} (h)	3.00 ± 1.16	5.00 ± 1.25	0.17 ± 0.00
AUC$_{(0-t)}$ (ng h/mL)	6355.89 ± 1707.42	6853.18 ±1385.74	30640.68 ± 10979.67
Cl/F (mL/h kg)	3795.57 ± 460.61	3526.87 ± 753.43	807.17 ± 368.87
V/F (mL/kg)	25876.90 ± 11689.90	10125.58 ± 1349.81	4911.50 ± 1607.60
Kel (h^{-1})	0.15 ± 0.06	0.35 ± 0.10	0.16 ± 0.09
$T_{1/2}$ (h)	4.72 ± 1.22	1.99 ± 0.42	4.22 ± 1.15
MRT (h)	6.49 ± 0.94	4.43 ± 0.42	3.07 ± 1.28
F (%)	20.74	22.37	

Each value represents the mean ± SD (n = 8). F represents absolute bioavailability.

following oral administration of PPD oil solution were similar to those of PPD solution. This may have been because PPD was also absorbed through the form of PPD molecule in PPD oil solution. But after oral administration of PPD emulsion, the $T_{1/2}$ value of PPD was reduced from 4.22 to 1.99 h, and the Kel value was increased from 0.16 to 0.35. One possible reason was the accelerated clearance of the emulsion from the body, which could be attributed to the particle size (micrometer level) of emulsion. So PPD emulsion was more prone to be taken up by RES in liver, spleen and kidney. Another possible explanation for this was that the emulsion could be metabolized by liver metabolism enzymes in the first pass effect, which would lead to the smaller $T_{1/2}$ value of PPD in PPD emulsion.

Differences in MRT between PPD solution (3.07 h), PPD emulsion (4.43 h) and PPD oil solution (6.49 h) may result from their different absorption phases and distribution phases.

Those results suggested that PPD emulsion was the best oral formulation, which showed strong and durative therapeutic effect. In spite of lower AUC than PPD emulsion, PPD oil solution was still a promising oral formulation for PPD. Because of its simple preparation, it is suitable as a PPD oil solution for the applications in industry production. PPD oil solution was administrated in a relatively low concentration of 2.5 mg/mL. Actually, 20 mg/mL of PPD could be also dissolved in tea-seed oil. Following the treatment of PPD oil solution at this concentration, the plateau plasma drug concentration would be further increased by much more effective formation of chyle and better lymphatic absorption.

Conclusions

A sensitive UPLC coupled to ESI-MS/MS method was developed and validated for the quantification of PPD in rat plasma after SPE. The described method was sensitive and accurate enough to determine PPD. The assay has been successfully applied to a pharmacokinetic study in rats following oral administration of PPD at a single dose of 25 mg/kg. The assay allows high sample throughput because of its simple sample preparation and short cycle time. Among the two oral PPD formulations, PPD emulsion appeared to have both the highest AUC and the most long-lasting plateau plasma drug concentration; PPD oil solution showed nearly the same duration of plateau plasma drug concentration with simple preparation and good promise for bioavailability enhancement. Both of the two PPD formulations displayed a long-acting effect *in vivo*.

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