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Short communication

Sensitive determination of 20(*S*)-protopanaxadiol in rat plasma using HPLC–APCI-MS: Application of pharmacokinetic study in rats

Hong-can Ren[a], Jian-guo Sun[a,**], Guang-ji Wang[a,*], Ji-ye A[a], Hai-tang Xie[a], Wei-bin Zha[a], Bei Yan[a], Fen-zhi Sun[a], Hai-ping Hao[a], Sheng-hua Gu[a], Long-sheng Sheng[a], Feng Shao[b], Jian Shi[a], Fang Zhou[a]

[a] *Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing 210038, PR China*
[b] *Laboratory of Drug Metabolism and Pharmacokinetics, Jiangsu Simcere Pharmaceutical R&D Co. Ltd., Nanjing 210042, PR China*

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ABSTRACT

20(*S*)-Protopanaxadiol (PPD), the main metabolite of protopanoxadiol type ginsenosides (e.g. Rg3 and Rh2), is a very promising anti-cancer drug candidate. To evaluate the pharmacokinetic property of PPD, we reported a reliable, sensitive and simple method utilizing liquid chromatography (HPLC)-atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) to determine PPD. PPD and the internal standard, panoxadiol (PD) were extracted from plasma with acetic ether, separated on a C18 reverse column, and then analyzed by APCI-MS. Targeting fragment ion at *m/z* 425 for both PPD and PD was monitored in selected-ion monitoring (SIM) mode. PPD can be quantitatively determined at the concentration as low as 1 ng/mL using 200 μL plasma. And the sensitive method showed excellent linearity over a range from 1 to 1000 ng/mL, high recovery, accuracy and precision at the concentrations of 2.5, 100.0 and 1000.0 ng/mL, respectively. The method was successfully applied to pharmacokinetic study of PPD in rats. Pharmacokinetic parameters were calculated and absolute bioavailability of PPD was 36.8 ± 12.4%, at least ten times higher than that of Rg3 and Rh2, indicating its good absorption in gastrointestinal tract. It was further suggested that PPD be a promising anti-cancer candidate and probably responsible for the observed pharmacological activity of Rg3 and Rh2.

1. Introduction

Ginsenosides are generally believed to be the main bioactive compounds found in ginseng [1]. Ginsenoside Rg3 and Rh2 (Rg3, Rh2) belong to protopanaxadiol (PPD) type ginsenosides share a similar basic structure, gonane steroid nucleus. Rg3 can be degraded into Rh2 and further into PPD by losing one or two attached sugar moieties in the gastrointestinal tract through a series of deglycosylation steps [2–4]. Structure–function relationship study of Rg3, Rh2 and PPD suggested that their anti-cancer bioactivity decreased as increasing number of the glycosidic attachment of sugar moieties either at C-3 or C-6 in the gonane structure [5]. Rg3-containing Shen-yi capsules have been approved for commercial market by Chinese State Food and Drug Administration (SFDA) for the treatment of cancer and Rh2 is being developed as another antitumor drug in China. Moreover, PPD exhibited powerful pleiotropic anti-cancer effects in several cancer cell lines and possessed the capability of inhibiting metastasis [6–8]. Further investigation discovered that PPD exerted its anti-cancer effect by inhibiting cell proliferation, altering membrane integrity, blocking cell cycle and inducing apoptosis [5,7,9]. However, little of Rg3 and Rh2 could be absorbed in gastrointestinal tract, so that it was inconsistent with their promising anti-cancer bioactivity orally [10,11]. Hence, it was hypothesized that the ginsenosides exerted their pharmacological effects through their mutually major metabolite, PPD [12]. As a powerful and promising drug candidate, pharmacokinetic study of PPD is not only necessary, but also a strong evidence to explain anti-cancer effect of Rg3 and Rh2. Due to the rather low dosage and plasma level pharmacokinetics/bioavailability of PPD have not been evaluated. In this paper, a sensitive method based on HPLC–APCI-MS was developed and applied to study the pharmacokinetics of PPD.

HPLC with UV detection, HPLC–ESI-MS and HPLC–ESI-MS/MS [10,11,13–27] have reported to determine ginsenosides in herbs and biological samples. However, few of them have been applied for pharmacokinetic study of PPD up to date. We had previously developed a LC–ESI-MS method for simultaneous determination of

* Corresponding author. Tel.: +86 25 85391035; fax: +86 25 85306750.
** Corresponding author.
E-mail address: guangjiwang@hotmail.com (G.-j. Wang).

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Rg3, Rh2 and PPD [10]. However, due to insufficient sensitivity the metabolite (PPD) kinetics of Rg3 could not be profiled comprehensively after oral administration of Rg3. Another report employed a HPLC–MS/MS method to determine 25-hydroxylprotopanoxadiol, an analogue of PPD, in rat plasma [13]. This method is also insufficiently sensitive (LOD, 10 ng/mL). In order to obtain a comprehensive profile of PPD to characterize its pharmacokinetics, we developed a sensitive HPLC–APCI-MS method, optimized the analytical parameters and validated the method.

2. Experimental

2.1. Chemicals and reagents

PPD, panoxadiol (PD), protopanaxatriol (PPT), panoxatriol (PT), and ginsenoside Rh2, Rh1, Rb, Rc, Rg3, Rd, Re, R1, Rg1, Rg2 and Rf (purity all over 99%) were purchased from Department of Nature Medical Chemistry, School of Chemistry, Jilin University, Changchun, China. Deionized water was prepared by Milli-Q system (Millipore, Bedford, MA, USA). Methanol was of HPLC grade (Merck, Darmstadt Germany). Ethyl acetate and all of other reagents, solvents were of analytical grade. Pure nitrogen gas was supplied by the Gas Supplier Center of Nanjing University, China.

2.2. Calibration standards and quality control (QC) samples

The stock solution of PPD (1.15 mg/mL) and PD solution (1.5 μg/mL) were prepared in methanol and stored in 4 °C. Calibration standard solutions were prepared at 1, 2.5, 5, 10, 25, 50, 100, 250, 500 and 1000 ng/mL of PPD in plasma, while QC samples were prepared at concentrations of 1, 2.5, 100 and 1000 ng/mL.

2.3. Sample preparation

In 2.0-mL polyethylene centrifuge tube, an aliquot of 200 μL plasma sample was added and spiked with 10 μL IS solution. After vortex-mixing for 10 s, 1.5 mL of ethyl acetate was added into each tube and the mixture was vigorously vortexed for 3 min. The organic and aqueous phases were separated by centrifugation at 8000 rpm for 5 min. One mL upper organic phase was transferred to another tube and evaporated to dryness at 40 °C using a Thermo Savant SPD 2010 Speed Vac System (Thermo Electron Corporation, USA). The residue was dissolved in 100 μL of the methanol, and vortex-mixed for 1 min. After centrifugation at 20,000 rpm for 10 min, 5 μL supernatant was injected into the HPLC–MS system for analysis.

2.4. Apparatus

A Shimadzu 2010A liquid chromatograph–mass spectrometer (Shimadzu, Kyoto, Japan) was utilized to carry out the analysis. The system consisted of two Shimadzu HPLC-10ADvp pumps, a Shimadzu SIL-HTc autosampler, a Shimadzu CTO-10Avp column oven, a Shimadzu DGU-14AM online degasser and a Shimadzu FCV-12AH flow channel selection valve. All data processing was performed using Shimadzu LCMSsolution (Version 2.04).

2.5. Chromatographic conditions and mass spectrometric conditions

Separation was performed using a Gemini C18 column (150 mm × 4.6 mm i.d., 5 μm, Phenomenex, Torrance, CA, USA) fitted with a C18 guard column (4.0 mm × 2.0 mm, 5 μm, Phenomenex, USA) at 40 °C with a mobile phase of methanol/water (95:5, v/v) at a flow rate of 0.75 mL/min. In APCI source, the probe was set at 4.0 kV and the temperature of the probe was kept at 380 °C. Other MS parameters were selected as followed: CDL (curved desolvation line) temperature, 250 °C; the block temperature, 200 °C; detector gain, 1.6 kV; CDL voltage, 5 V; Q-array DC (direct current) voltage, 0 V. Nitrogen served as nebulizer gas (flow rate: 2.5 L/min) and curtain gas (pressure: 10 kPa). Mass spectra were obtained at a dwell time of 0.2 and 1 s in SIM and scan mode, respectively. Masses were scanned from m/z 300 to 700, where the major fragment ions were detected. In SIM mode positive ions of PPD and PD were all monitored at m/z 425.

2.6. Validation of the method

Linearity of PPD over the range from 1 to 1000 ng/mL was investigated with PD as the IS. The extraction recovery of PPD were calculated by comparing the peak area of analyte dissolved in blank plasma extract with that dissolved in the mobile phase. To evaluate intra-day and inter-days' accuracy and precision, QC samples at concentrations of 1, 2.5, 100, 1000 ng/mL ($n = 5$) were analyzed at different time points within the same day or over five consecutive days. Accuracy of the method was calculated by the equation: (determined concentration/actual concentration) × 100% and the precision was expressed as CV. QC samples at concentrations of 2.5, 100, 1000 ng/mL were applied 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 1 week, (3) keeping at 4 °C for 24 h, the same condition as that in autosampler.

2.7. Pharmacokinetic study of PPD in rats

Animal studies were carried out in accordance with the Chinese National Research Council guidelines and were approved by the Subcommittee on Research Animal Care and Laboratory Animal Resources of China Pharmaceutical University. Sprague–Dawley male rats (Sino-British Sippr/BK Lab Animal Ltd., Shanghai, China) weighing 200–220 g were used to study the pharmacokinetics of PPD and acclimated in a standard animal laboratory with a 12 h light–dark cycle. PPD was dissolved in saline with hydroxypropyl-β-cyclodextrin to increase the solubility. After overnight fast (12 h), rats were intravenously given with PPD via caudal vein at a dose of 0.2 mg/kg within 1 min or administrated 2 mg/kg PPD by oral garage. Water was freely accessible, but no food was allowed until the rat had been given the drug for 3 h. Blood samples were collected by orbital sinus bleeding at 0 (prior to dosing), 0.033, 0.16, 0.5, 1, 2, 3, 5, 8, 12 and 24 h after the i.v. injection and at 0 (prior to dosing), 0.25, 0.5, 1, 1.5, 2, 4, 8, 12 and 24 h after oral administration. After centrifugation at 4000 rpm for 5 min, plasma was separated and stored at −80 °C until analysis. Plasma PPD concentration versus time data for each rat was analyzed by DAS software (Ver 1.0, Medical College of Wannan, China). 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 $[(\mathrm{AUC_{i.g.}}) \times (\mathrm{Dose_{i.v.}})]/[(\mathrm{AUC_{i.v.}}) \times (\mathrm{Dose_{i.g.}})] \times 100\%$.

3. Results

3.1. APCI-MS analysis and optimization of MS parameters

At early stage of the developing the method, electrospray ionization (ESI) was applied for the quantification of Rg3, Rh2, and PPD [10]. The major ions observed were the adduct ion of chloride $[M+CL]^-$. APCI-MS was only applied to identify the ginsenosides because the method could provided more structure information [28]. To our surprised, the analysis with APCI source in scan mode resulted very high MS responses. The

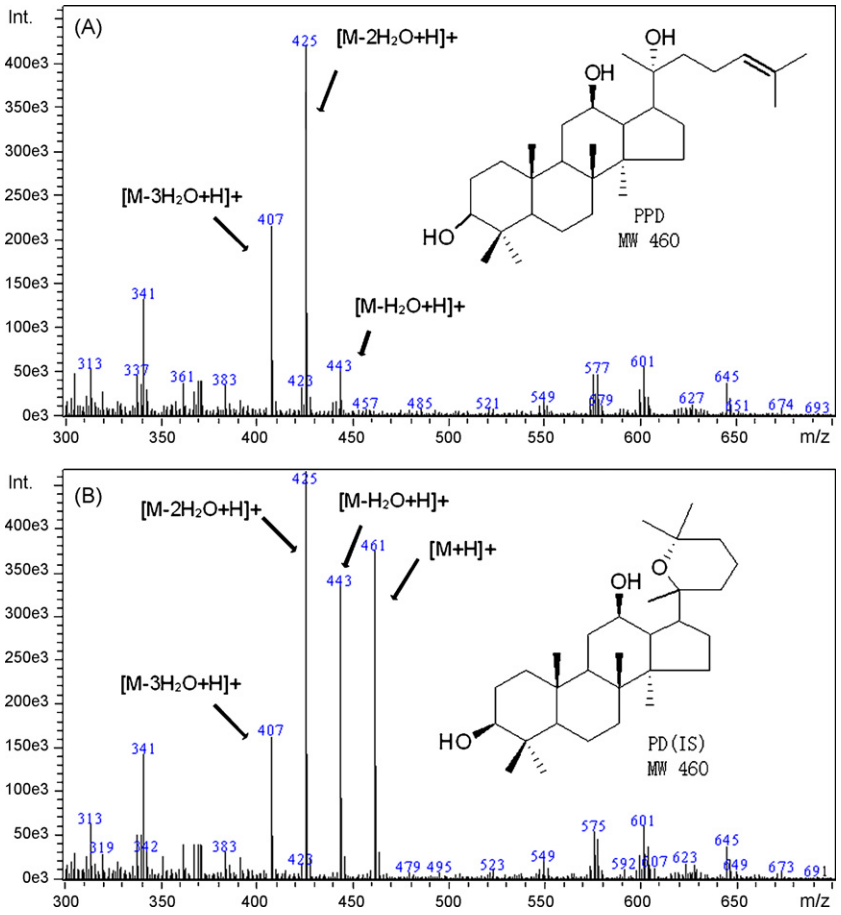

Fig. 1. Mass spectra of PPD and PD in scan mode with APCI (+) source. (A) PPD. (B) PD.

highest intensity and good reproducibility was observed at *m*/*z* 425, ions of $[M-2H_2O+H]^+$, which were selected for the selected-ion monitoring (SIM) throughout the following study (Fig. 1). APCI parameters were optimized with probe temperature ranging from 300 to 500 °C and probe voltages from 2.5 to 4.5 kV. It was found that a combination of probe temperature at 380 °C and probe voltage on 4.0 kV yielded best sensitivity.

3.2. Choice of IS

As previously reported, to select an ideal IS for the analysis, 14 ginsenosides were analyzed with the same APCI-MS setting. Judged by similarity of molecular structure, sensitivity, retention time, and matrix interferences, protopanaxatriol (PPT), Rh2 and PD were found the best IS candidates for further evaluation. PPT was rejected for its low recovery using acetic ether. Rh2 seemed the best because of its appropriate retention time and high recovery efficiency. However, Rh2 was not very stable in acidic environment and can be converted into PPD. To avoid possible interferences Rh2 was not chosen. As the consequence, PD was added in all of the samples despite of it's a bit longer retention time. In addition, it was suggested that the presenting method with APCI source might be a possible choice for the determination of the 14 ginsenosides, although the mobile phase has to be modified to determine different ginsenosides so that the retention times become suitable.

3.3. Assay specificity

The specificity of the method was investigated by comparing MS chromatograms of PPD with that of blank plasma, sample spiked with PPD and a plasma sample from a rat at 10 min after i.v. administration of PPD. In the APCI mode, blank rat plasma yielded clean chromatograms without significant interference both to PPD and IS, while peaks of PPD and IS were shown sharp and steady in the plasma samples (Fig. 2).

3.4. Linearity and limit of quantification

Good linearity was observed with the coefficients of determination (r^2) ranged from 0.991 to 1.000 as $y = 0.003042x - 0.000078$, where y indicates the peak area ratios of PPD to IS, and x indicates the plasma PPD concentration. The limit of quantification (LOQ) was 1 ng/mL for PPD with the signal-to-noise of over 10. In our previous study, the LOQ of PPD was 8 ng/mL by HPLC–ESI-MS [10]. Since plasma level of PPD in rat declined very rapidly and was not able to be detected in 6 h after i.v. administration, the sensitivity of previous method could not meet the requirement of the pharmacokinetic study of PPD in rats. With the developed method by LC-APCI-MS, the LOQ reached as low as 1 ng/mL, more sensitive than that by HPLC–ESI-MS. The improved sensitivity facilitated determination of PPD in rat plasma over a period of 12 h after a single i.v. dose of 0.2 mg/kg PPD so that its pharmacokinetic parameters could be approached.

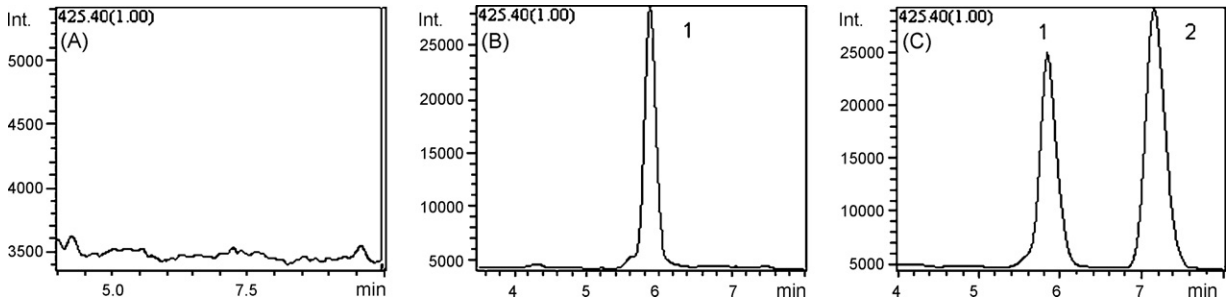

Fig. 2. MS chromatograms of (A) blank rat plasma. (B) Blank rat plasma spiked with PPD (50 ng/mL). (C) Rat plasma sample collected 5 min after i.v. injection of 0.2 mg/kg PPD. (1) PPD; (2) IS.

Table 1
Inter-day and intra-days precision and accuracy of PPD in plasma.

Spiked concentration (ng/mL)	Intra-day ($n = 5$)			Inter-day ($n = 5$)		
	Determined concentration (mean ± S.D.) (ng/mL)	CV (%)	Accuracy (%)	Determined concentration (mean ± S.D.) (ng/mL)	CV (%)	Accuracy (%)
1.0	0.89 ± 0.09	9.6	89.0	1.11 ± 0.12	10.9	110.5
2.5	2.45 ± 0.08	3.3	97.8	2.65 ± 0.29	10.9	106.0
100.0	111.26 ± 4.33	3.9	111.3	106.99 ± 5.67	5.3	107.0
1000.0	1011.13 ± 35.92	3.6	101.1	949.50 ± 73.16	7.7	94.9

3.5. Recovery

The absolute recoveries of PPD were 71.1, 93.8 and 85.7%, while the relative recoveries of PPD were 78.2, 85.2 and 84.9% at 2.5, 100.0 and 1000.0 ng/mL, respectively.

3.6. Precision, accuracy and stability of the samples

The coefficients of variation (CV) for all samples at three levels were less than 10.9% and the accuracy ranged from 89.0 to 111.3% (Table 1). The stability of PPD in plasma indicated that PPD was quite stable under three conditions simulating those in storage, during sample preparation, and in autosampler. No degradation (<10%) occurred in each condition.

3.7. Pharmacokinetic study of PPD

The presented method was successfully applied to quantify PPD in rat plasma following a single bolus 0.2 mg/kg i.v. dose or after oral administration 2 mg/kg PPD. Fig. 3 shows the mean plasma concentration–time curves in rats after i.v. (Fig. 3A) or oral (Fig. 3B) administration. Main pharmacokinetic parameters of PPD are shown in Table 2. The PPD concentration–time data after intravenous dosing was best fitted to a two-compartment open model

Table 2
Mean pharmacokinetic parameters of PPD after i.v. injection of 0.2 mg/kg and oral administration of 2 mg/kg in five SD rats (mean ± S.D.).

Parameter	i.v. injection (0.2 mg/kg)	Oral administration (2 mg/kg)
$T_{1/2ka}$ (min)	–	54.8 ± 13.9
T_{max} (min)	–	150.0 ± 73.5
C_{max} (μg/mL)	–	0.1 ± 0.0
CL/F (mL/(min kg))	21.2 ± 2.3	–
V/F (mL/kg)	1030.9 ± 611.4	1087.4 ± 818.7
$T_{1/2\alpha}$ (min)	7.3 ± 4.4	–
$T_{1/2\beta}$ (min)	163.9 ± 57.1	88.6 ± 17.8
$AUC_{(0-tn)}$ (μg/mL min)	8.5 ± 1.5	33.8 ± 12.0
$AUC_{(0-\infty)}$ (μg/mL min)	9.5 ± 1.1	34.3 ± 11.9
$MRT_{(0-tn)}$ (min)	124.9 ± 22.6	208.5 ± 35.1
$MRT_{(0-\infty)}$ (min)	274.3 ± 108.8	219.2 ± 39.5

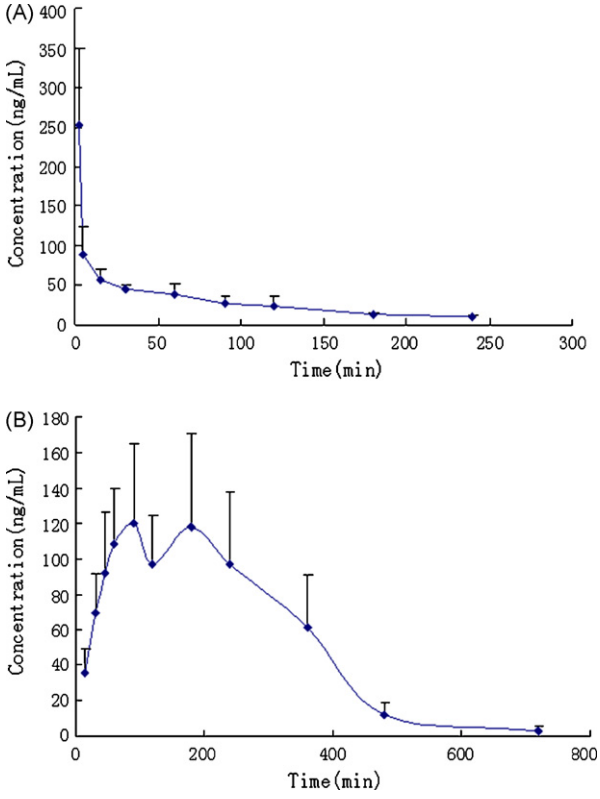

Fig. 3. Plasma concentration–time curve of PPD following an i.v. injection of 0.2 mg/kg (A) and oral dose of 2 mg/kg (B) to rats. Data are presented as mean ± S.D. ($n = 5$).

based on Akaike's Information Criterion (AIC). It was characterized by a rapid falling followed by a relative slower decrease in the terminal phase after intravenous administration of 0.2 mg/kg PPD. As for oral administration, the concentration–time data was fitted best to a one-compartment model. C_{max} of PPD administrated orally was 130.2 ± 41.5 ng/mL corresponding mean T_{max}

value was 150.0 ± 73.5 min. The absolute bioavailability reached as high as 36.8 ± 12.4% indicating good absorption of PPD in gastrointestinal tract. It was reported that the absolute bioavailability of Rg3 was 2.63%, while absolute bioavailability was so low that no Rh2 could be detected in plasma after oral administration of it at 100 mg/kg. The very low bioavailability of Rg3 and Rh2 was inconsistent with their unambiguous pharmacological effects and puzzled us for a long time. Since most of Rg3 and Rh2 was metabolized into PPD in the gastrointestinal tract [2–4] and PPD can be absorbed well here, it was indicated that Rg3 and Rh2 exerted their activities through their mutual active metabolite, PPD.

4. Conclusion

A novel HPLC–APCI-MS method was developed, optimized and validated to determine PPD in rat plasma. The described method was proven sensitive, robust and accurate to determine PPD. Owing to this improved detection sensitivity, the whole elimination phase of PPD was profiled either after i.v. administration of PPD at 0.2 mg/kg, or after oral administration 2 mg/kg PPD. We believe that this developed method could also be further applied for study of metabolite kinetics after administration of Rg3 or Rh2. The absolute bioavailability of PPD was 36.8 ± 12.4%, at least ten times higher than that of Rg3 and Rh2, which makes PPD an attractive candidate for the therapeutic treatment of cancer orally. The results of this study indicated that PPD might be responsible for the observed pharmacological activity of Rg3 and Rh2.

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