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Short communication

Rapid and sensitive LC–MS/MS assay for the quantitation of 20(S)-protopanaxadiol in human plasma

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ABSTRACT

This paper describes a rapid and sensitive method for the quantitation of 20(S)-protopanaxadiol (PPD) in human plasma based on high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS). The analyte and internal standard (I.S.), ginsenoside Rh_2, were extracted from plasma by liquid–liquid extraction and separated on a Zorbax extend C_{18} analytical column using methanol–acetonitrile-10 mM ammonium acetate (47.5:47.5:5, v/v/v) as mobile phase. Detection was by tandem mass spectrometry using electrospray ionization in the positive ion mode and multiple reaction monitoring (MRM). The assay was linear over the concentration range 0.1–100.0 ng/ml with a limit of detection of 0.05 ng/ml. The method was successfully applied to a clinical pharmacokinetic study in healthy volunteers after a single oral administration of a PPD 25 mg capsule.

1. Introduction

20(S)-Protopanaxadiol (PPD) (dammar-24-ene-3β,12β,20S-triol) is the aglycone of PPD-containing ginsenosides such as ginsenoside Rb1, Rb2, Rb3, Rc, Rd, Rg3 and Rh2. It is a metabolite of some ginsenosides [1–4] and also the product of their acid or alkaline hydrolysis [5,6]. PPD has antitumour activity and is a potential therapeutic agent in the prevention and treatment of cancer [7–11]. As such it is currently undergoing phase I clinical trials in China.

As part of its preclinical assessment, we have studied the pharmacokinetics of radiolabelled PPD in rats and dogs and found that its oral bioavailability is very low due to extensive metabolism in the gastrointestinal tract. Accordingly, further study of its pharmacokinetics in human at therapeutic doses (25 mg) requires a sensitive analytical method. Since the structure of PPD contains only a weak chromophore, it is a prime candidate for analysis by liquid chromatography mass spectrometry (LC–MS).

Although LC–MS methods have been developed for the assay of PPD, they are confined to its determination as a metabolite of ginsenoside Rh2 in rat [12] and of Rg3 in rat [2,3] and dog [13]. These methods determine PPD simultaneously with its parent ginsenoside and suffer from long analysis time and relatively low sensitivity (e.g. 8.0 ng/ml [3]). Clinical pharmacokinetic studies of PPD in human have not been reported. In this paper we describe a rapid and sensitive LC–MS assay for the determination of PPD in human plasma and its successful application to a clinical pharmacokinetic study of PPD in healthy volunteers.

2. Experimental

2.1. Chemicals and reagents

PPD (99.8%) and ginsenoside Rh_2 (98.8%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing, China). Yijinsheng Capsules for use in the pharmacokinetic study were produced and supplied by the Asian Pharmaceutical Group (Hainan, China). Methanol and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Distilled water was prepared from deionized water. All other chemicals and solvents (analytical grade) were used without further purification. Blank (drug free) human plasma was obtained from the Changchun Blood Donor Service (Changchun, China).

2.2. Instrumentation

The HPLC system was an Agilent Model 1100 series (Agilent, Palo Alto, CA, USA) consisting of a pump, autosampler and column

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oven set at 40 °C. An API-4000 tandem mass spectrometer (Applied Biosystems/MDS SCIEX, Concord, Ontario, Canada) equipped with an electrospray ionization (ESI) source was used for mass analysis and detection. Analyst software (Applied Biosystems/MDS SCIEX, version 1.3) installed on a *DELL* computer was used for data acquisition and processing.

2.3. LC–MS/MS conditions

Chromatography was carried out on a C18 analytical column (Zorbax extend, 150 mm × 4.6 mm i.d., 5 μm, Agilent, USA) with a mobile phase of methanol, acetonitrile and 10 mM ammonium acetate (47.5:47.5:5, v/v/v) at a flow rate of 1.3 ml/min. An approximately 1:1 split of the column eluant was made and hydrophilic impurities were diverted to waste for 100 s after injection using a 10-way switching valve.

The mass spectrometer was operated in the positive ion mode. Detection by multiple reaction monitoring (MRM) used the transitions at *m/z* 461.6 → 425.5 and *m/z* 461.6 → 443.5 for PPD and at *m/z* 623.5 → 605.5 for ginsenoside Rh_2. Other parameters were as follows: collision gas, curtain gas, gas 1 and gas 2 (nitrogen) 5, 10, 40 and 25 p.s.i., respectively; dwell time 200 ms; IonSpray voltage 4800 V; source temperature 200 °C; declustering potential (DP) 38 V for 20(S)-PPD and 36 V for Rh_2; collision energy (CE) 15 eV (*m/z* 461.6 → 425.5) and 10 eV (*m/z* 461.6 → 443.5) for 20(S)-PPD and 10 eV for Rh_2. Low resolution was used for both Q1 and Q3 mass detection. The MS/MS conditions and analyte response were optimized by introducing a standard solution of analyte via a syringe pump at 20 μl/min.

2.4. Preparation of calibration standards and quality control samples

Stock solutions (400 μg/ml) of PPD and I.S. were prepared by dissolving 10.0 mg in 2 ml methanol followed by dilution to 25 ml with methanol. Low, medium and high PPD quality control (QC) solutions (0.3, 10, and 80 ng/ml) were prepared by diluting the stock solution with water. An I.S. working solution (80 ng/ml) was prepared by diluting the stock solution with methanol. All solutions were stored at 4 °C when not in use.

A calibration standard (100 ng/ml) was prepared by serial dilution of the stock solution with blank plasma. Further calibration standards (0.1, 0.3, 1, 3, 10, and 30 ng/ml) were prepared by dilution of the 100 ng/ml calibration standard with blank plasma. All calibration standards were freshly prepared. QC samples (0.3, 10, and 80 ng/ml) were prepared independently in a similar manner. All QC samples were prepared in bulk, aliquoted, and frozen at −20 °C, with aliquots thawed and analyzed with each run.

2.5. Sample preparation

Frozen plasma samples were thawed at room temperature and vortex-mixed briefly. An aliquot of plasma (500 μl) was placed in a glass tube followed by I.S. working solution (100 μl) and 3 ml diethyl ether:dichloromethane (60:40, v/v). The mixture was vortex-mixed for 90 s, shaken for 10 min, and centrifuged at 3500 × *g*, room temperature (about 18 °C) for 5 min. The organic phase was transferred to another clean glass tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 150 μl mobile phase and 40 μl injected into the LC–MS system.

2.6. Assay validation

The method was fully validated based on FDA guidelines [14]. Specificity was assessed by assay of pooled blank plasma from six healthy volunteers. Matrix effects for analyte and I.S. were investigated by comparing the peak areas of post-extraction blank plasma samples spiked with analyte and I.S. with mean peak areas of post-extraction spiked water at the same concentrations. Linearity was assessed by linear regression of calibration curves based on peak area ratios of analyte to I.S. weighted according to $1/x^2$ (x = concentration). Accuracy and precision were determined by assay of six replicates of each QC sample on three separate days each with an independently prepared calibration curve. The lower limit of quantitation (LLOQ) was defined as the concentration below which the relative error (R.E.) was >±15% or the inter-day relative standard deviation (R.S.D.) exceeded 15%. The limit of detection (LOD) was defined as the concentration with signal-to-noise ratio of three. Recovery of analyte and I.S. was determined by comparing the peak areas of analyte and I.S. in extracted QC samples with mean peak areas of analyte and I.S. reconstituted in blank plasma

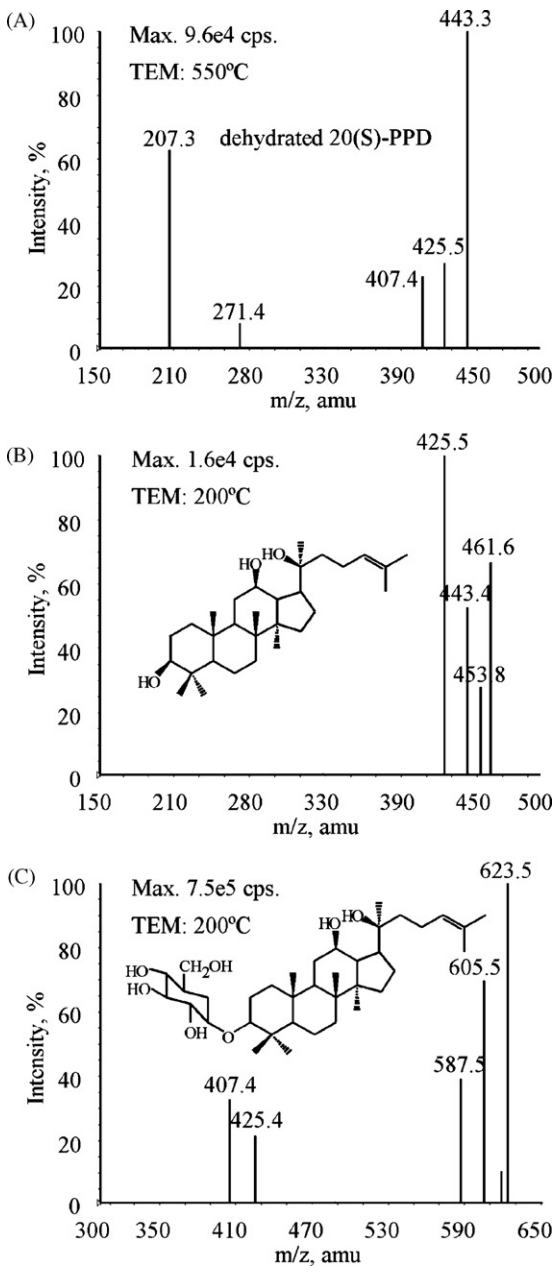

Fig. 1. Full-scan product ion spectra of $[M+H]^+$ for (A) dehydrated 20(S)-protopanaxadiol in source (B) 20(S)-protopanaxadiol and (C) ginsenoside Rh_2 (TEM = source temperature).

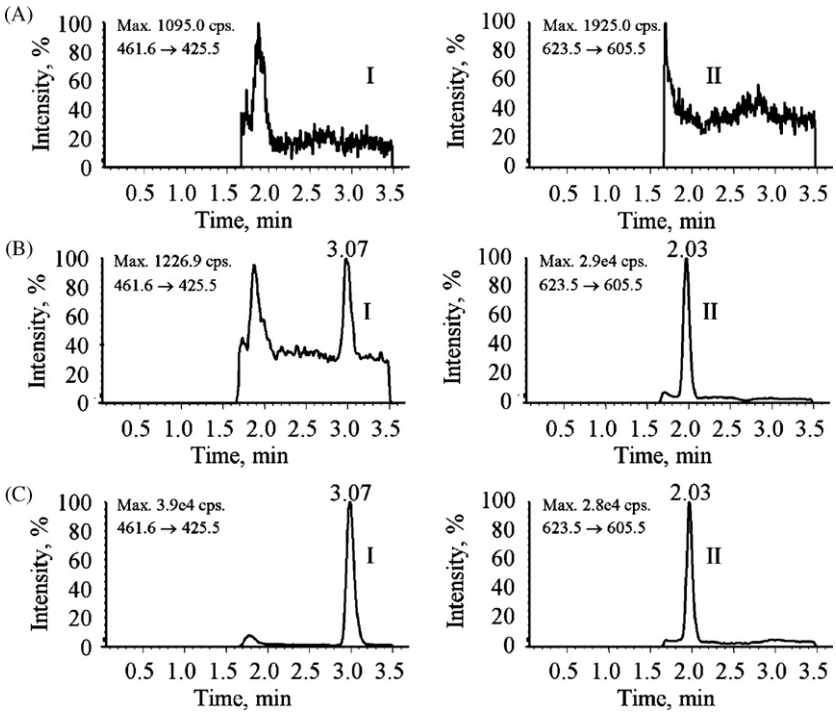

Fig. 2. Representative MRM chromatograms of (A) blank plasma; (B) plasma spiked with 20(S)-protopanaxadiol at the lower limit of quantitation (0.1 ng/ml) and (C) plasma sample from a healthy volunteer 1 h after a single oral administration of a capsule containing 25 mg 20(S)-protopanaxadiol. Peak I, 20(S)-protopanaxadiol; Peak II, ginsenoside Rh_2.

extracts at the corresponding concentrations. Stability of PPD was evaluated in QC samples stored at −20 °C for 60 days, after three freeze/thaw cycles and after storage at room temperature for 6 h. Stability of PPD in reconstitution solutions in the autosampler at room temperature for 6 h was also determined.

2.7. Pharmacokinetic study

The validated assay was applied to a pharmacokinetic study in eight healthy volunteers (four males, four females) aged 30–40 years, with body mass index (BMI) 19–24. The study was approved by the Ethics Committee of the Affiliated Hospital of the Academy of Military Medical Sciences, Beijing, P.R. China. Volunteers were selected after completing a thorough medical, biochemical and physical examination. All volunteers gave informed consent after the aims and risks of the study were fully explained.

Volunteers were given a single oral dose of a PPD 25 mg capsule with 250 ml water. Blood samples (2 ml) were collected by venepuncture into heparinized tubes before and 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24 h after the oral dosing. Plasma was separated by centrifugation (3000 × *g* for 10 min at 4 °C) and stored immediately at −20 °C until analysis. All samples were analyzed within 60 days. Pharmacokinetic parameters were calculated using Topfit 2.0.

3. Results and discussion

3.1. LC–MS/MS conditions

The response of analyte to ESI was greater in the positive ion mode. In the full-scan mode at a source temperature of 550 °C, the ion produced by dehydration at *m/z* 443.4 gave the greatest response and using it as precursor ion produced an abundant transition at *m/z* 443.4 → 207.3 (Fig. 1A). However, at a source temperature of 200 °C, the parent ion at *m/z* 461.6 gave the greatest response and, in the assay of plasma samples, the transition at *m/z* 461.6 → 425.5 gave better selectivity and specificity with adequate sensitivity. The product ion mass spectra of analyte (*m/z* 461.6) and I.S. using a source temperature of 200 °C are shown in Fig. 1B and 1C respectively. In the mass spectrum of analyte (*m/z* 461.6), the product ion at *m/z* 425.5 was more abundant than that at *m/z* 443.4 leading to adoption of the transition *m/z* 461.6 → 425.5 for quantitation and *m/z* 461.6 → 443.4 as qualifier.

Several types of commercial reversed-phase HPLC columns (Zorbax, Venusil, Nucleosil, Hypersil and Restek) and various mobile phase compositions were investigated to optimize resolution and peak shape in the minimum cycle time. Chromatography on a Zorbax extend C_{18} column with a mobile phase of methanol–acetonitrile–10 mM ammonium acetate (47.5:47.5:5, v/v/v) at 1.3 ml/min gave good separation of PPD and I.S. with minimal matrix effects in a short cycle time of 3.5 min.

3.2. Assay validation

Fig. 2 shows typical MRM chromatograms of blank plasma, plasma spiked with PPD at 0.1 ng/ml, and a plasma sample from a healthy volunteer 1 h after oral administration of a 25 mg PPD capsule. The assay was shown to be free of interference from endogenous substances in plasma. In terms of matrix effects, the ratios of the peak responses for PPD were 90.1 ± 1.5%, 88.9 ± 4.9%, and 92.0 ± 4.3% at 0.3, 10, and 80 ng/ml, respectively. The response for I.S. was 96.0 ± 7.1%. The results indicated that co-eluting endogenous substances inhibited the ionization of PPD and I.S slightly, however, the ion suppression from plasma matrix was consistent for this analytical method and would not interfere the measurement of PPD. Data on relative matrix effects is shown in Table 1.

Linear calibration curves with correlation coefficients (r) > 0.9979 were obtained in the concentration range 0.1–100 ng/ml. The LLOQ of 0.1 ng/ml was sufficient for clinical pharmacokinetic studies following oral administration of a 25 mg dose of PPD. The LOD of PPD was determined to be 0.05 ng/ml. A summary of inter- and intra-day precision and

Table 1
The matrix effect of PPD at three QC concentration levels and of IS at the working concentration.

PPD				
		Concentration (ng/mL)		
		0.300	10.0	80.0
Mean peak area of 6 matrixes (n = 3)	Matrix A	3.87E+03	7.45E+04	5.69E+05
	Matrix B	3.82E+03	7.42E+04	5.72E+05
	Matrix C	3.92E+03	7.79E+04	6.24E+05
	Matrix D	3.81E+03	7.67E+04	6.30E+05
	Matrix E	3.97E+03	8.12E+04	6.27E+05
	Matrix F	3.92E+03	8.54E+04	6.15E+05
Mean peak area of neat solution (n = 3)		4.31E+03	8.81E+04	6.59E+05
Matrix effect (%)	Matrix A	89.8	84.6	86.3
	Matrix B	88.6	84.2	86.8
	Matrix C	91.0	88.4	94.7
	Matrix D	88.4	87.1	95.6
	Matrix E	92.1	92.2	95.1
	Matrix F	91.0	96.9	93.3
Matrix effect(%)		90.1	88.9	92.0
R.S.D (%) (n = 6)		1.5	4.9	4.3
IS				
Working concentration (80.0 ng/mL)		LQC	MQC	HQC
Mean peak area of 6 matrixes (n = 3)	Matrix A	1.30E+05	1.32E+05	1.25E+05
	Matrix B	1.11E+05	1.31E+05	1.23E+05
	Matrix C	1.11E+05	1.14E+05	1.07E+05
	Matrix D	1.26E+05	1.20E+05	1.19E+05
	Matrix E	1.02E+05	1.09E+05	1.18E+05
	Matrix F	1.26E+05	1.15E+05	1.24E+05
Mean peak area of neat solution (n = 9)			1.24E+05	
Matrix effect (%)	Matrix A	104.8	106.5	100.8
	Matrix B	89.5	105.6	99.2
	Matrix C	89.5	91.9	86.3
	Matrix D	101.6	96.8	96.0
	Matrix E	82.3	87.9	95.2
	Matrix F	101.6	92.7	100.0
Matrix effect (%)			96.0	
R.S.D (%) (n = 18)			7.1	

accuracy at the LLOQ and the three QC concentrations is shown in Table 2. Precision was <9.0%. and accuracy within ±2.0% at all concentrations. The recoveries of PPD from human plasma were 83.3 ± 4.5%, 81.6 ± 1.3%, and 89.5 ± 2.7% at concentrations of 0.3, 10, and 80 ng/ml, respectively. The recovery of I.S. was 96.6 ± 4.6%. In terms of stability, the changes in concentration of PPD under the various storage conditions examined (summarized in Table 2) indicate there was no significant decomposition of PPD.

3.3. Clinical pharmacokinetic study in healthy volunteers

This assay was applied to a clinical pharmacokinetic study in healthy volunteers following a single oral administration of a PPD 25 mg capsule. The mean plasma concentration-time profiles are shown in Fig. 3. The pharmacokinetic parameters for PPD are as follows: C_{max} 7.24 ± 3.30 ng/ml; T_{max} 1.28 ± 0.49 h; $t_{1/2}$ 4.77 ± 2.05 h; AUC_{0-t} 23.4 ± 13.2 ng h ml^{-1}; $AUC_{0-\infty}$ 25.0 ± 13.8 ng h ml^{-1}; CL/F 23.4 ± 11.0 l h^{-1} kg^{-1}; V_d/F 158 ± 111 l kg^{-1}.

Table 2
Precision and accuracy for the determination of PPD in human plasma (Data are based on assay of six replicate samples at the LLOQ and three QC concentrations on three different days).

Nominal concentration (ng/ml)	Measured concentration (mean ± SD, ng/ml)	Inter-day RSD (%)	Intra-day RSD (%)	RE (%)
0.100	0.101 ± 0.004	2.23	3.75	1.00
0.300	0.296 ± 0.009	2.76	5.06	−1.33
10.0	10.2 ± 0.9	8.74	4.19	2.00
80.0	80.3 ± 5.2	6.81	2.11	0.38

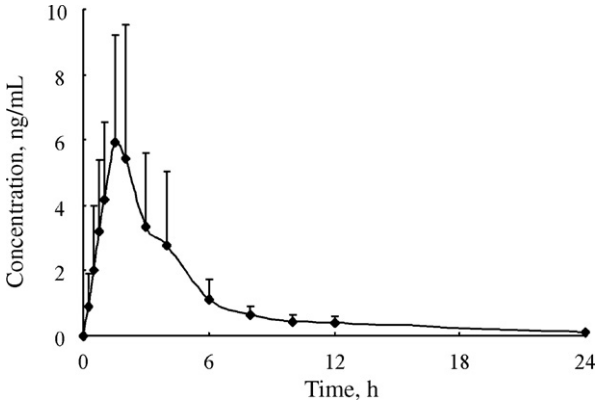

Fig. 3. Mean plasma concentration–time profile of 20(S)-protopanaxadiol after a single oral administration of a 25 mg capsule to healthy volunteers. Data are means + SD (n = 8, 4 males, 4 females).

4. Conclusions

A rapid and sensitive LC–MS/MS method for the determination of 20(S)-protopanaxadiol in human plasma is reported. The assay

has been successfully applied to a clinical pharmacokinetic study in healthy volunteers following a single oral administration of a 25 mg capsule. The assay allows high sample throughput because of its simple sample preparation and short cycle time.

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