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Journal of Chromatography B, 858 (2007) 65–70
JOURNAL OF CHROMATOGRAPHY B
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Determination of 25-OH-PPD in rat plasma by high-performance liquid chromatography–mass spectrometry and its application in rat pharmacokinetic studies

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Received 15 May 2007; accepted 8 August 2007
Available online 23 August 2007

Abstract

A sensitive and specific liquid chromatography/tandem mass spectrometry (LC/MS/MS) method was developed for the investigation of the pharmacokinetics of 20(*R*)-dammarane-3β,12β,20,25-tetrol (25-OH-PPD) in rat. Ginsenoside Rh_2 was employed as an internal standard. The plasma samples were pretreated by liquid–liquid extraction and analyzed using LC/MS/MS with an electrospray ionization interface. The mobile phase consisted of methanol–acetonitrile–10 mmol/l aqueous ammonium acetate (42.5:42.5:15, v:v:v), which was pumped at 0.4 ml/min. The analytical column (50 mm × 2.1 mm i.d.) was packed with Venusil XBP C_8 material (3.5 μm). The standard curve was linear from 10 to 3000 ng/ml. The assay was specific, accurate (accuracy between −1.19 and 2.57% for all quality control samples), precise and reproducible (within- and between-day precisions measured as relative standard deviation were <5% and <7%, respectively). 25-OH-PPD in rat plasma was stable over three freeze–thaw cycles and at ambient temperatures for 6 h. The method had a lower limit of quantitation of 10 ng/ml, which offered a satisfactory sensitivity for the determination of (25-OH-PPD) in plasma. This quantitation method was successfully applied to pharmacokinetic studies of 25-OH-PPD after both an oral and an intravenous administration to rats and the absolute bioavailability is 64.8 ± 14.3%.

Keywords: 25-OH-PPD; LC/MS/MS; Pharmacokinetics

1. Introduction

Ginsenosides and their aglycones have long been used for medical purposes in treatment of cancer, diabetes, and heart problems [1]; there is increasing interest in developing ginseng products as cancer preventive or therapeutic agents [2–4]. The main ginsenosides are glycosides that contain an aglycone with a dammarane skeleton and include protopanaxadiol-type saponins, such as ginsenosides Rb_1, Rb_2, Rb_3, Rc and Rd, and protopanaxatriol-type saponins such as ginsenosides Re and Rg_1 Rg_2, Rh_1. Among the saponin ginsenosides are compounds with a dammarane structure, of which there are two main classes: panaxadiols (PPD) and panaxatriols (PPT).

Wang et al. [5] determined biological structure–activity relationships for 11 saponins present in *Panax* ginseng fruits with their in vitro cytotoxicity against several human cancer cell lines. It was first reported a dammarane structure, compound of 20-dammarane-3, 12, 20, 25-tetrol (25-OH-PPD) (Fig. 1) had been shown to inhibit tumor metastasis in mice and in vitro tumor cell invasion. 25-OH-PPD is aglycone of PPD-type ginsenoside and has the same basic structure; the difference is the variation in their side-chains. It is an effectively inhibitors of cell growth and proliferation and inducers of apoptosis and cell cycle arrest. 25-OH-PPD had significant, dose-dependent effects on apoptosis, proliferation, and cell cycle progression. 25-OH-PPD, the IC_{50} values for most cell lines were in the range of 10–60 μM, demonstrating a 5–15-fold greater growth inhibition than Rg_3.

An investigation of the pharmacokinetics and bioavailability of compound can link data from pharmacological assays to clinical effects and also help in designing rational dosage regimens. Further studies are needed to characterize the bioavailability and pharmacokinetics of 25-OH-PPD in order to fully take advantage

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doi:10.1016/j.jchromb.2007.08.021

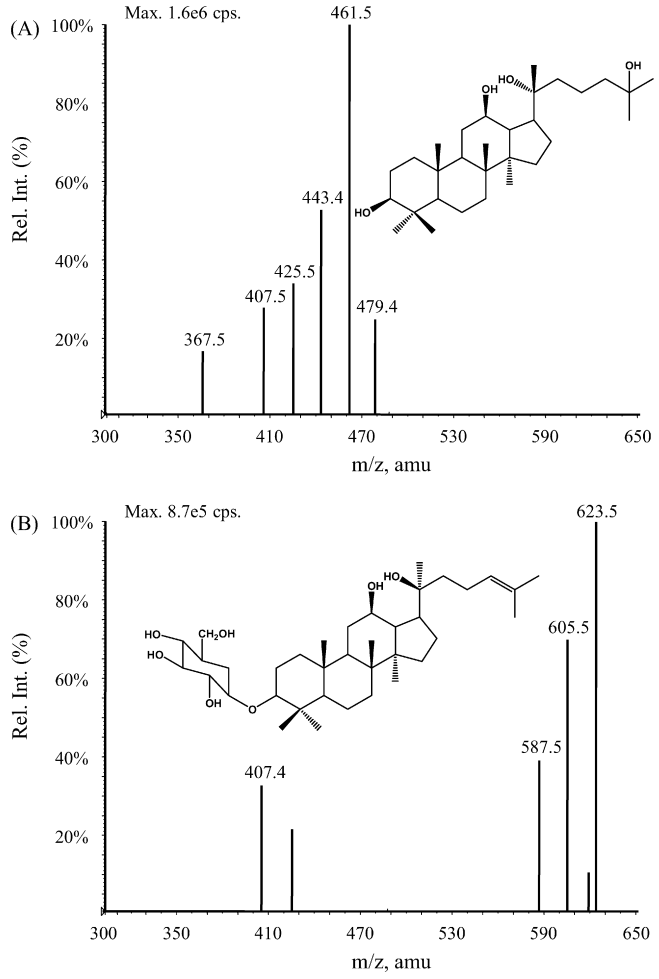

Fig. 1. Product ion mass spectra of $[M+H]^+$ ions of (A) 25-OH-PPD and (B) ginsenoside Rh_2, (IS).

of the notable pharmacodynamics activity. The pharmacokinetics and metabolism of ginsenosides to rats, dogs or human have been performed and the results suggest that ginsenosides are very poorly absorbed following oral administration in vivo [6–19]. There are a few reports on LC/MS/MS analysis of ginsenosides extracted from biological samples [8–19]. Up to now, the determination of 25-OH-PPD has not been reported. The establishment and application of sensitive and reproducible method of determining 25-OH-PPD in rat plasma by LC/MS/MS method is presented in this paper.

2. Materials and methods

2.1. Materials

25-OH-PPD (99.0% pure) was supplied by Shenyang Pharmaceutical University (Liaoning, China) and 20(*R*)-ginsenoside Rh_2 (internal standard, IS, 98.8% purity) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP). Acetonitrile and methanol (HPLC grade) were purchased from Tianjin Kangkede Technology Co. Ltd. (Tianjin, China). Water was doubly distilled in the laboratory. All other chemicals were purchased from commercial sources and used as received.

2.2. Chromatography and mass spectrometry

The high-performance liquid chromatography was performed on an Agilent 1100 system (Palo Alto, CA, USA) equipped with a G1313A autosampler, a vacuum degasser unit, and a G1312A binary pump. The mobile phase consisted of methanol–acetonitrile–10 mM aqueous ammonium acetate (42.5:42.5:15, v/v/v), without the adjustment of pH, delivered at a flow rate of 0.4 ml/min. The injection volume was 10 μl. The analytical column used was packed with Venusil XBP C_8 material (50 mm × 2.1 mm i.d., 3.5 μm) from Agela, USA. The chromatography was performed at 40 °C. The HPLC system was coupled in line to an API 4000 mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) via a TurboIonSpray ionization interface. Following optimization of the setting, the instrument was operated in the positive mode with an ion spray voltage of 0.5 kV, curtain gas pressure of 15 psi, nebulizer gas pressure of 40 psi, heater gas pressure of pressure of 45 psi, collision gas pressure of 5 psi, and the source temperature was set at 250 °C. The curtain, nebulizer, heater and collision gases were all nitrogen. The fragmentation transitions for the multiple reaction monitoring (MRM) were *m/z* 479.4 → 443.4 amu for 25-OH-PPD, and *m/z* 623.5 → 605.5 amu for Rh_2. Data were collected and analyzed by the Analyst 1.3 Data Acquisition and Processing software (Applied Biosystems/MDS Sciex).

2.3. Preparation of stock and working solutions

The stock solution of 25-OH-PPD (400 μg/ml) was prepared in methanol–water (1:1, v/v) and serially diluted to give working solutions of 5, 15, 50, 150, 500, and 1500 ng/ml in methanol–water (1:1, v/v). A 500 ng/ml solution of the internal standard was similarly prepared by diluting 400 μg/ml stock solution of ginsenoside Rh_2 in methanol–water (1:1, v/v). All stock solutions and working solutions were stored at 4 °C.

2.4. Preparation of calibration standards and quality control (QC) samples

Calibration standards and QC samples of 25-OH-PPD were prepared by spiking 100 μl of the working solutions and 100 μl of Rh_2 to 50 μl of drug-free rat plasma. Calibration standards were prepared at concentrations of 10, 30, 100, 300 1000 and 3000 ng/ml of 25-OH-PPD in plasma. QC samples at 30, 300, and 2400 ng/ml of 25-OH-PPD used in the validation were prepared as described above, then QC samples and calibration standards were treated as described in Section 2.5.

2.5. Sample treatment

A volume of 100 μl of the IS, 100 μl of methanol/water (1:1, v/v) were added to 50 μl of plasma from the rats dosed orally or by i.v. injection. This mixture was extracted with 3 ml of ether–dichloromethane (3:2, v/v) by shaking for 10 min. The

organic and aqueous phases were separated by centrifugation at 3500 × *g* for 5 min. The upper organic phase was transferred to another tube and evaporated to dryness at 40 °C under a gentle stream of air. The residue was dissolved in 200 μl of the mobile phase, and vortex-mixed for 1 min. A 10 μl aliquot of the solution was injected onto the LC/MS/MS system for analysis.

2.6. Method validation

Standard curves ranging from 10 to 3000 ng/ml of 25-OH-PPD were run on 3 separate days. The integrated ion chromatogram peak areas of 25-OH-PPD and Rh_2 were used to construct a standard curve from the peak area ratio versus nominal 25-OH-PPD concentration using linear regression analysis with $1/x^2$ weighting. Six replicates of QC samples at 30, 300, and 2400 ng/ml of 25-OH-PPD were included in each run to determine the within- and between-run precision of the assay by performing the complete analytical runs on the same day and also on three consecutive days. The accuracy was determined as a percent difference between the mean detected concentrations and the nominal concentrations. The relative standard deviation (RSD) was used to report the precision. The data from these QC samples were examined by a one-way analysis of variance (ANOVA). The lower limit of quantitation (LLOQ) was assessed by analyzing 18 plasma samples spiked with 10 ng/ml of 25-OH-PPD in three runs and the lowest concentration was on the standard curve.

The matrix effect was evaluated by comparing the response of the solution spiked with the blank processed matrix with the solution at the same concentration. Absolute recoveries of 25-OH-PPD at three QC levels were determined by assaying the samples as described above and comparing the peak areas of both 25-OH-PPD and IS with those obtained from direct injection of the compounds dissolved in the supernatant of the processed blank plasma.

2.7. Stability

The stability of 25-OH-PPD in the plasma was assessed by analyzing triplicate QC samples at 30, 300, and 2400 ng/ml stored for 6 h at ambient temperatures, following three cycles of freezing at −20 °C and thawing and also following 30 days at −20 °C. Concentrations following storage were compared to freshly prepared samples of the same concentrations.

2.8. Application of the method

Wistar rats (220 ± 20 g), used in the pharmacokinetics study of 25-OH-PPD, were purchased from the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). All experimental procedures carried out in this study were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Shenyang Pharmaceutical University in Shenyang. Rats were administered 10 mg/kg 25-OH-PPD by oral administration after an overnight fasting period or intravenous injection of 5 mg/kg to Wistar rats via the tail vein. The drug was formulated by dissolving 25-OH-PPD with a dimethyl sulfoxide–PEG 400–physiologic saline (1:4:10, v:v:v) and mixed well. Blood samples (150 μl) were collected into heparinized tubes from each rat by the puncture of the retroorbital sinus. This was performed at 0 (pre-dose), 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h after administration orally and at 0 (pre-dose), 0.033, 0.16, 0.5, 1, 2, 3, 4, 5, 8, 12 and 24 h after i.v. administration via the tail vein. Blood was immediately processed for plasma by centrifugation at 3000 × *g* for 10 min. Plasma samples were frozen and maintained at −20°C until analysis to six Wistar rats via the tail vein.

Plasma-concentration data for individual dogs were analyzed by non-compartmental analysis using the TopFit 2.0 software package (Thomae GmbH, Germany). Maximum plasma concentration (C_{max}) and the time-to-maximum concentration (T_{max}) were estimated by visual inspection of semi-logarithmic plots of the concentration–time curves. The area under the curve (AUC_{0-t}) was calculated using the linear-trapezoidal rule, with extrapolation to infinity ($AUC_{0-\infty}$) from the last detectable concentration using the terminal elimination rate constant (k_e) calculated by linear regression of the final log-linear part of the drug concentration–time curve. Apparent elimination half-life ($t_{1/2}$) was calculated as $t_{1/2} = 0.693/k_e$, total body clearance (CL) as dose/$AUC0_{0-\infty}$, and apparent volume of distribution (Vd) as CL/k_e. Absolute bioavailability was determined from the ratio of dose normalized AUC_{0-24} values obtained for oral versus i.v. administered drug which was expressed as $(F\ \%) = (Dose_{oral})$ (AUC)/$(Dose_{i.v.})$ (AUC).

3. Results and discussion

3.1. LC–MS/MS optimization

The responses of 25-OH-PPD and Rh_2 to ESI were evaluated by recording the full-scan mass spectra in both positive and negative ionization modes, introducing 25-OH-PPD and Rh_2 solutions via a syringe pump. The protonated mode yielded a signal higher for the deprotonated molecule of 25-OH-PPD (*m/z* 477.4) compared with the response for the molecule (*m/z* 479.4) in the negative mode. The signal for Rh_2 (*m/z* 621.5) in the negative mode was similar to that (*m/z* 623.5) in the positive mode, and over two-fold lower than the signal for 25-OH-PPD (*m/z* 479.4).

The fragmentation transitions for the multiple reaction monitoring were *m/z* 479.4 → 443.4 amu for 25-OH-PPD, and *m/z* 623.5 → 605.5 amu for Rh_2. These MS/MS fragmentations are shown in Fig. 1. For the detection of Ginsenoside Rh_2 the transition involves the loss of water, it is not interacted with endogenous material at this high concentration 500 ng/ml.

Although the structure of ginsenoside Rh_2 is different from 25-OH-PPD, it was chosen as IS. The reason is that Rh_2 and 25-OH-PPD are the derivates of protopanoxadiol. The satisfactory peak shape and similar retention time with that of 25-OH-PPD were got under the chromatographic conditions.

During the optimization of chromatographic conditions, columns packed with different types of C_{18} or C_8 material (Nucleosil, Hypersil, Zorbax) were tried; 25-OH-PPD and ginsenoside Rh_2 (IS) were extensively retained on these columns.

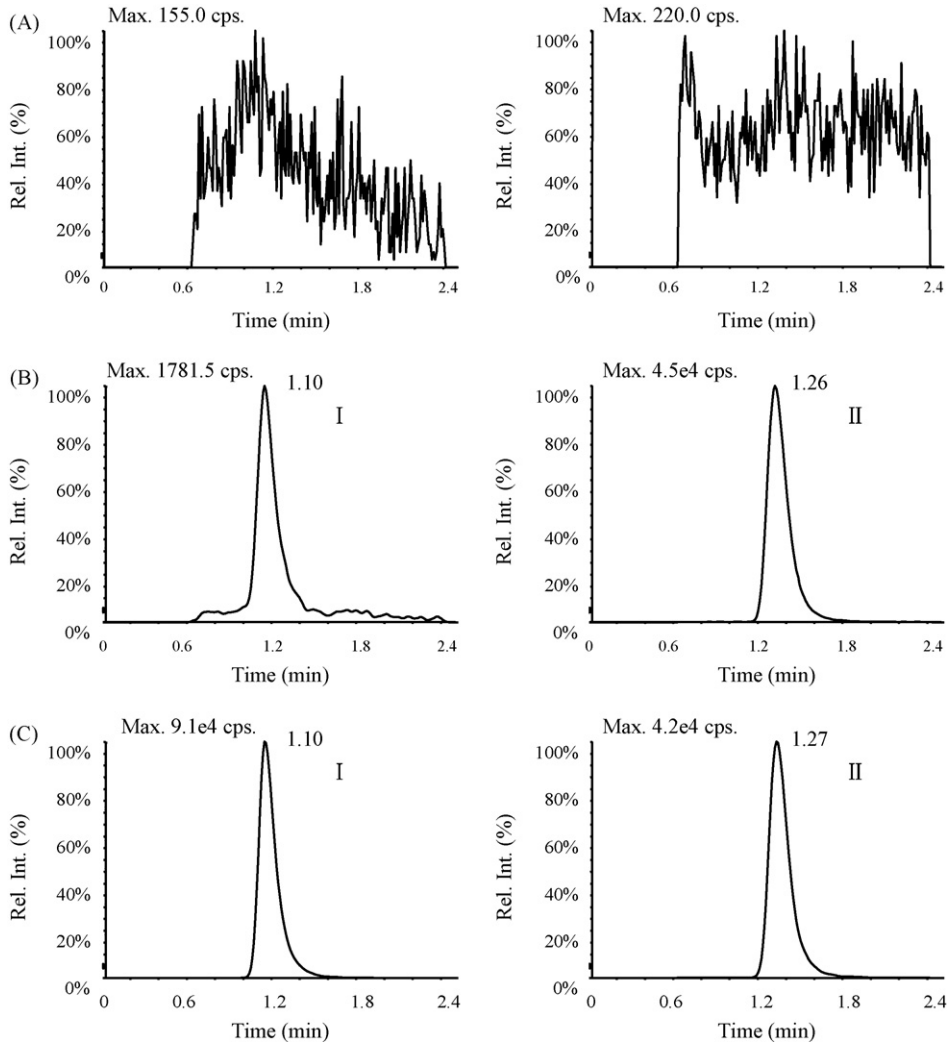

Fig. 2. MRM chromatograms (I, *m/z* 479.4 → *m/z* 443.4 amu; II, *m/z* 623.5 → 605.5 amu) for (A) drug-free plasma, (B) plasma spiked with 10 ng/ml of 25-OH-PPD (LLQ) and 500 ng/ml Rh_2 and (C) a plasma sample 1 h after the oral administration to a rat with 10 mg/kg of 25-OH-PPD. I represented 25-OH-PPD; II represented Rh_2.

To achieve symmetrical peak shapes and short chromatographic cycle times, a mobile phase consisting of methanol-acetonitrile-10 mM aqueous ammonium acetate (42.5:42.5:15, v/v/v) was used, with the 50 mm Venusil XBP C_8 column.

3.2. Sensitivity and specificity

The specificity of the method was demonstrated by comparing MRM chromatograms for 25-OH-PPD and IS. Rh_2 for a drug-free plasma sample, a spiked plasma sample, and a plasma sample from a rat 1.0 h after oral administration. As shown in Fig. 2, no significant peaks interfering with analytes were observed in the drug-free rat plasma. The retention times for 25-OH-PPD and the internal standard were approximately 1.1 and 1.27 min, respectively. The present LC/MS/MS method offered an LLOQ of 10 ng/ml with an accuracy of −2.9% and a precision of 7.8% ($n = 6$). This LLOQ are sufficient for pharmacokinetic studies of 25-OH-PPD in rat.

Matrix effects from co-eluting endogenous substances provide another possible source of problems regarding assay specificity, although matrix-matched calibration standards were used. The ion suppression effect was evaluated by comparing the peak areas of 25-OH-PPD in QC samples (30, 300, and 2400 ng/ml) and Rh_2 the IS (500 ng/ml) with those of standard solutions that had been prepared in the same way as the QC samples except that water was substituted for drug free plasma. The ratios of the peak responses were 103.6 ± 5.4%, 100.8 ± 5.7% and 105.9 ± 5.3% at 30, 300, 2400 ng/ml for analyte and 95.6 ± 2.0% for IS, respectively. These observations indicate that no endogenous substances significantly influenced the ionization of these analytes.

The recovery of 25-OH-PPD, determined at three concentrations (30, 300, 2400 ng/ml), were 87.4 ± 2.8%, 96.3 ± 2.1% and 97.2 ± 0.8% ($n = 6$), respectively. The recovery of Rh_2 was investigated as 96.8 ± 3.4% ($n = 6$).

For 25-OH-PPD, the mean peak areas from the six QC samples had relative errors of 4.0%, and the degree of intensity ranged from 0.8 to 5.9% when compared with that for these standard solutions. For the IS, the relative error was −4.6%, and the degree of suppression ranged from 3.9 to 7.6%. These obser-

Table 1
Precision and accuracy results for 25-OH-PPD in rat plasma (3 days, six replicates per day)

Plasma concentration (ng/ml)	Mean measured concentration (ng/ml)	Accuracy (%)	Within-run precision (%)	Between-run precision (%)
30.0	30.4	1.2	3.1	2.9
300.0	296.4	−1.2	4.5	5.4
2400.0	2461.0	2.6	2.4	6.5

Table 2
Stability data for 25-OH-PPD at different storage condition ($n = 3$)

Stability test	Mean concentration (ng/ml)		Recovery (%)
	Before storage	After storage	
Stability after three freeze–thaw cycles	30.6	30.8	100.9
	287.0	299.6	104.4
	2327.1	2417.3	103.9
Stability in rat plasma at ambient temperature for 6 h	30.6	31.8	103.8
	301.7	303.0	100.4
	2507.0	2550.0	101.7
Stability in rat plasma at −20 °C for 30 days	31.1	31.4	100.9
	288.7	307.0	106.3
	2390.0	2530.0	105.9

vations indicate that no endogenous substances significantly influenced the ionization of these analytes.

3.3. Validation

The correlation coefficients for the standard curves ranged from 0.9972 to 0.9994. The validation concentration range was from 10 to 3000 ng/ml, using 50 μl of plasma. The assay performance for the determination of 25-OH-PPD is shown in Table 1. The accuracies for all tested concentrations were within 10% of nominal and both the within- and between-run precisions were acceptable [20].

3.4. Stability

A several stability experiments were performed and the results are summarized in Table 2. No significant changes in the 25-OH-PPD concentrations were measured after three freeze–thaw cycles, storage at −20 °C for 30 days and storage for 6 h at ambient temperature.

3.5. Application of the method

The presented method was successfully applied to quantify 25-OH-PPD in the plasma of rats for 24 h following a single 10 mg/kg oral dose ($n = 4$) or after i.v. 5 mg/kg ($n = 4$). The concentration versus time profiles after oral and i.v. administration are shown in Fig. 3. The main pharmacokinetic parameters of 25-OH-PPD after oral and i.v. administration calculated by non-compartmental analysis are shown in Table 3.

As for oral administration, the mean C_{max} value was 4617 ± 1571 (range 2950–6560 ng/ml), corresponding mean T_{max} value was 5.5 ± 4.7 h (range 1.0–12.0 h). The mean plasma elimination half-life was 3.9 ± 2.0 h (range 2.7–7.0 h). As for i.v. administration elimination half-life was 4.5 ± 2.5 h (range 2.9–8.3 h). Absorption of 25-OH-PPD from rat gastrointestinal tract was rapid and retained for a long period of time in the plasma after oral administration. The plasma concentration–time curves of the compound exhibited distinct double-peaks after oral administration and this might involve enterohepatic recirculation. The identification of enterohepatic recirculation may be required by a comparison of AUC obtained after oral

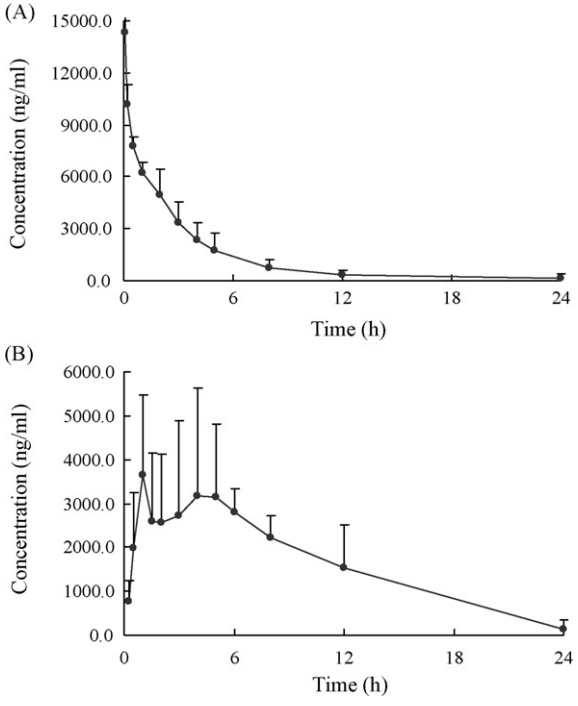

Fig. 3. Mean rats plasma concentration–time profiles of 25-OH-PPD after (A) an oral dose of 10 mg/kg and (B) an i.v. dose of 5 mg/kg (each point represents mean ± SD, $n = 4$).

Table 3
The main pharmacokinetic parameters of 25-OH-PPD after oral (10 mg/kg) and i.v. (5 mg/kg) administrations to four rats (mean ± SD)

Parameter	Administration mode	
	Oral (10 mg/kg)	Intravenous (5 mg/kg)
C_{max} (ng/ml)	4617.2 ± 1571.3	
T_{max} (h)	5.5 ± 4.7	
$t_{1/2}$ (h)	3.9 ± 2.1	4.5 ± 2.6
k_e (1/h)	0.2 ± 0.1	0.2 ± 0.1
AUC_{0-t} (ng h/ml)	38954 ± 5172	31808 ± 11685
$AUC_{0-\infty}$ (ng h/ml)	40194 ± 6666	33487 ± 14834
MRT (h)	8.1 ± 3.1	4.7 ± 2.9
V_d/F (ml/kg)	1355.4 ± 510.6	950.2 ± 115.0
CL/F (ml/h/kg)	254.1 ± 42.7	166.6 ± 52.4

administration of the compound in normal and bile duct cannulated rats. The absolute bioavailability is 64.8 ± 14.3% (range 44.1–75.9%) which is the highest among the reported in ginseng compounds and it is very beneficial to drug exerting in clinical application at antitumor activity in the future. Pharmacokinetic studies suggest that ginsenosides are very poorly absorbed following oral administration to rats and humans and ginseng saponin metabolites formed by intestinal bacteria were identified after oral administration of ginseng extracts in humans and rats [14,6–11,14,21–23]. The results suggested that the natural ginsenoside may be the prodrug and the hydrolysis products, such as ginsenoside Rh_2 and protopanaxadiol could play an important role in the therapeutic activities. Xu et al. reported the absolute bioavailability of Rg1 was 18.4% in rats [6] and Li et al. reported the absolute bioavailability was 15.62% for Rg1, 0.28% for Rb1 and 0.34% for Rd [7]. It was reported the absolute bioavailability of ginsenoside Rg3 in rats was 2.63% [9] or undetectable in oral dosing samples [8]. One of the major anticarcinogenic effect metabolites known as IH901 appears in the plasma after oral administration of the ginsenoside Rb1 and the absolute bioavailability values for the IH901 powder, the physical mixture, and the inclusion complexes were 3.52, 4.34, and 6.57%, respectively [12]. Peak reported the absolute bioavailability was 35.0% for a ginseng saponin metabolite compound K at the 20 mg/kg dose [14]. Wang et al. [19] reported the absolute bioavailability of Rd in dogs was 0.26%. The higher absolute bioavailability is found in the rats and it could be hypothesized that 25-OH-PPD possesses deglycosylated mother aglycone structure, lower molecular weight, higher hydrophobic molecule than that of ginsenoside Rg3 and so well absorbed by digestive tract due to GI stability. It was shown the standard deviation was quite big among individual rat (in Fig. 3). Further studies are needed to elucidate the absorption 25-OH-PPD at dose-linearity.

4. Conclusion

In conclusion, the present optimized method was validated to guarantee a reliable determination of 25-OH-PPD in rat plasma. It was then successfully applied to a pharmacokinetic study of 25-OH-PPD after both oral and intravenous administrations. It is the first report of LC/MS/MS method on the determination of 25-OH-PPD concentration in vivo so far. From the results of the present experiment, it seems reasonable to draw the conclusion that 25-OH-PPD could be absorbed through GI tract in rat after oral administration, and oral absolute bioavailability of 25-OH-PPD was relatively high than other ginsenosides such as Rb_1 and Rg_1. Both of them indicate that current administering routines, oral administration and even intravenous injection, are very appropriate. Further studies of absorption, distribution, excretion and metabolism of 25-OH-PPD are underway on rats and dogs in our laboratory. Analytical methods, including this assay described here, will be applied in these studies.

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