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LC–MS/MS quantification of 20(S)-protopanaxadiol in complex biological matrices for bioanalytical method validation and pharmacokinetic analysis

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20(S)-Protopanaxadiol (PPD) is a saponin derivative of ginsenoside, with more potent biological and pharmacological activities than Rg3 and Rh2. The lack of ionizable centers leads to low mass spectrometry reactions and internal cleavage of three hydroxyl groups, making it challenging to establish highly sensitive PPD mass spectrometry methods. The aim of this study is to establish and validate a quantitative detection method for PPD in multiple matrices using mass spectrometry. The methods used Rh2 as the internal standard and organic solvent liquid–liquid extraction under alkaline conditions for biological sample pretreatment. Isometric separation was achieved through methanol, acetonitrile, and a 10 mmol/L solution of acetic acid (45:45:10, v/v/v) at a flow rate of 0.4 mL/min. Finally, perform mass spectrometry quantification. Comprehensive method validation was conducted on rat plasma samples, and partial method validations were performed on three types of rat tissues (adipose tissue, smooth muscle, and skeletal muscle), bile, urine, fecal samples, and dog plasma samples. The results were in accordance with the requirements of NMPA for bioanalytical method validation, ensuring the accuracy and reliability of our analytical measurements. This study employed a conventional liquid–liquid extraction sample pretreatment scheme, utilizing multiple biological matrices commonly found in a single treatment protocol and liquid chromatography-tandem mass spectrometry detection parameters. The consistency of processing and detection across diverse samples eliminated the need for methodological changes, providing exceptional convenience. Up to 90% of the organic phase and a 50 mm short chromatographic column achieved rapid and effective separation of PPD. A key aspect of our work is the use of a "programmed injection" technique, which significantly reduces the analysis time from 4.2 min during method exploration to 2.4 min. These methods have achieved a relatively low quantification limit of 2.5 ng/mL. The methods established were successfully applied to the kinetic process of PPD in rats, and the pharmacokinetic characteristics of PPD in dogs were studied for the first time.

Keywords 20 (S)-protopanaxadiol, LC–MS/MS, Pharmacokinetic, Biological matrices, Programmed injection

Ginseng has been widely and historically used in traditional Chinese medicine, exhibiting various biological and pharmacological activities on the central nervous, cardiovascular, endocrine, and immune systems. Dammarane saponins and their metabolites are the main sources of its pharmacological activities[1,2]. Dammarane saponins isolated from Panax ginseng can be classified into two categories based on their aglycone moiety: protopanaxadiol (ginsenosides Ra1, Rb1, Rb2, Rb3, Rg3, Rh2, Rc, Rd) and protopanaxatriol (ginsenosides Re, Rg1, Rg2, Rh1)[3]. Protopanaxadiol saponins Rg3 and Rh2 exhibit inhibitory effects on tumor cell proliferation and growth both

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in vitro and in vivo and induce differentiation and apoptosis[4,5]. 20(S)-Protopanaxadiol (PPD) is the aglycone of protopanaxadiol saponins (Fig. 1) and has shown stronger pharmacological activity compared to Rg3 and Rh2[6,7]. However, only a few studies on the pharmacokinetics and metabolism of ginsenosides in rats and humans have been conducted[7–10], and and comprehensive and systematic study has not yet been conducted on the in vivo kinetic processes (absorption, distribution, metabolism, excretion) of PPD in rats and dogs.

The methods for determining ginsenosides, panaxadiol, and PPD in biological samples mainly include UV detection-based HPLC[7], fluorescence detection[11], GC–MS[12], and liquid chromatography-tandem mass spectrometry (LC–MS/MS)[8,9,13–19]. The focus of the above LC–MS/MS quantitative detection methods is different. For example, Zhao XE et al. focuses on introducing the dual ultrasound assisted dispersion liquid–liquid microextraction combination[14], Hong-Can Ren et al. uses APCI source[16], Yuanwu Bao uses lithium adducts as precursor ions[17], and Wenyan Wang et al. focuses on analyzing the isomers[18]. The above LC–MS/MS methods only focus on supporting quantitative detection in rat plasma, or human plasma and urine. In actual preclinical studies, in addition to blood, multiple matrix quantitative tests such as various tissues, urine, feces, and bile are commonly conducted. Consequently, the PPD quantitative detection method, which is applicable to multiple biological matrices, holds significant application value.

In this study, we conducted LC–MS/MS methodological development and validation of PPD in rat plasma, multiple tissue supernatant, bile, urine, fecal supernatant, and dog plasma to support accurate determination of PPD for pharmacokinetic study in complex matrices.

Materials and methods

Reagents, materials, and experimental animals

PPD (Lot No.: 20021006, purity: 98.8%) and internal standard ginsenoside Rh2 (Lot No.: 20000906, purity: 98.5%) were provided by Zhejiang Yake Pharmaceutical Co, Ltd (Zhejiang, China). Acetonitrile and methanol are both chromatographically pure and purchased from Fisher Scientific International Inc (Waltham, Mass, USA) and Tianjin Concord Technology Co, Ltd (Tianjin, China), respectively.

10 mg of PPD reference standard was weighed and prepared into a 1 mg/mL stock solution with methanol. The working curve and quality control samples (QCs) were diluted with matrix to the required concentration. The internal standard was 500 ng/mL Rh2 methanol–water mixture (1:1, v/v).

Wistar rats (220–270 g) from Shenyang Pharma Uni's Animal Lab and Beagle dogs (8-12 kg) from Military Med Sci's Animal Center were used. Experiments complied with Chinese Physiological Society's lab animal guidelines and received ethics approval from Jilin Uni's Lab Animal Ethics Committee, conducted and presented according to the ARRIVE guidelines[20]. Animals were not fasted or sedated for any drug administration or blood collection events.

LC–MS/MS quantitative analysis method

Chromatographic analysis was done with an Agilent 1100 HPLC system (Agilent Technologies, USA) and Zorbax C18 column (50 × 2.1 mm, 3.5 μm). Mobile phase: methanol, acetonitrile, and a 10 mmol/L solution of acetic acid (45:45:10, v/v/v), flow rate: 0.4 mL/min, column temp: 40℃.

MS analysis used API 4000 triple quadrupole mass spectrometer with ESI and Analyst 1.3 (Applied Biosystems). Ion spray: 4800 V, temp: 320 °C. Nitrogen flows: sheath 276 kPa, nebulize 173 kPa, curtain 69 kPa, collision 28 kPa. Positive ionization, multiple reaction monitoring (MRM) with DP voltage 35 V. Quant ions: m/z 461.6→425.5 (PPD), m/z 623.50→605.5 (IS Rh2), as in Fig. 1.

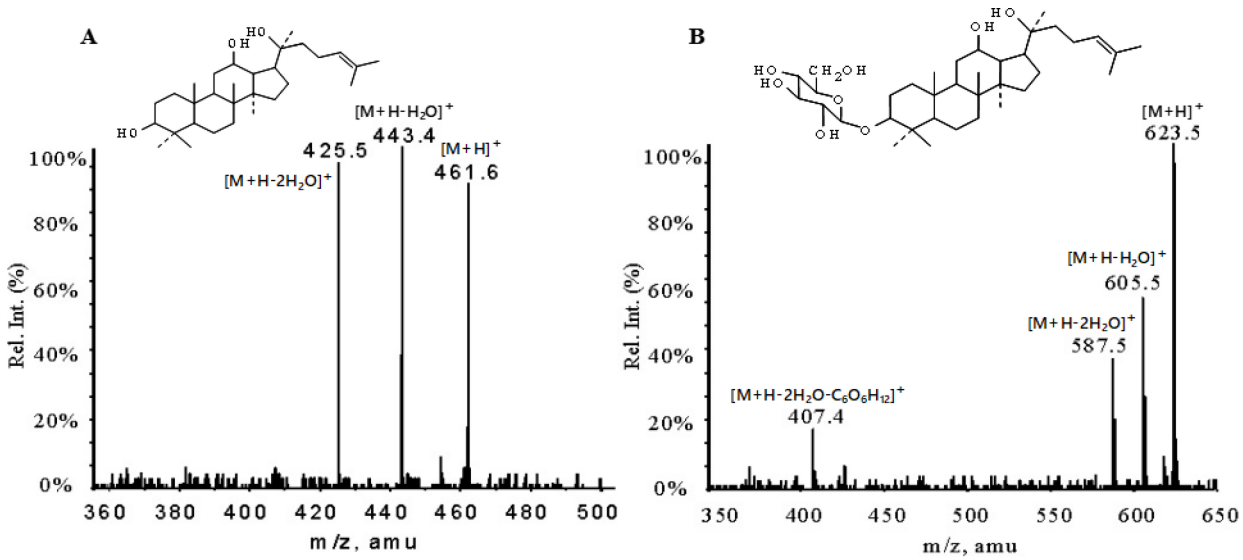

Fig. 1. Full-scan product ion spectra of $[M+H]^+$ for (**A**) PPD and (**B**) ginsenoside Rh2.

The biological sample (50 μL of rat plasma, dog plasma, fecal supernatant, or 100 μL of tissue supernatant, bile or urine) was mixed with 100 μL of a methanol–water mixture (1:1, v/v), 100 μL of internal standard solution (500 ng/mL Rh2), and 50 μL of sodium hydroxide solution (0.3 mol/L). Add 3 mL of ether-dichloromethane (3:2, v/v) for liquid–liquid extraction. The mixture was vortexed (1 min) and shaken (15 min). Then, it was spun at 3000 rpm for 5 min. The clear top liquid was dried under warm air (40℃). The leftovers were mixed in 300μL of mobile phase, and 20μL was injected for LC–MS/MS analysis.

Validation of LC–MS/MS method

This study conducted methodological development and validation of PPD in rat plasma, tissue supernatant, bile, urine, fecal supernatant, and dog plasma, with full validation (selectivity, calculation curve, accuracy, precision, recovery, matrix effect, and stability) for rat plasma and partial validation for others. The analytical method was validated within the guidelines established by the National Medical Products Administration, P.R. China (NMPA) for bioanalytical method validation.

The selectivity of the bioanalytical method included the interference between blank samples and analytes. Each analysis method compared six separate blank samples, including and excluding analytes and internal standard, to investigate potential interference peaks at retention time. The responses of analytes in the blank should not exceed 20% of lower limit of quantification (LLOQ) and 5% of the average internal standard (IS) responses of the calibrators and QCs, respectively.

The calculation curves prepared in the biological samples (rat plasma, tissue supernatant, bile, urine, fecal supernatant, and canine plasma) by spiking same matrix with standard solutions and validated in at least three batches. The calculation curves were produced by plotting the concentration of the standards against the analyte to IS peak area ratios using least square linear regressions with a weighting factor of $1/x^2$. The acceptance criteria involved coefficient of determination (r) ≥ 0.99 and RSD ≤ 15% at each concentration (LLOQ ≤ 20%).

Accuracy and precision of the method were evaluated by analyzing QCs at low, medium, and high concentrations. Six replicates of each concentration were analyzed for three consecutive days. Accuracy was expressed as the percentage of nominal concentration, of which acceptance criterion was within ± 15% of the target concentration (LLOQ within ± 20%). Precision was demonstrated as the coefficient variation (%CV), which should be ≤ 15% at each level (≤ 20% at LLOQ level).

The extraction recoveries at three different concentrations were determined by comparing the measured concentrations after extraction to those obtained without extraction. The matrix effect of analytes were evaluated by comparing the responses of the post-extraction six different blank spiked samples (B, corresponding internal standard response is BIS) with those of the neat solutions at three QC concentration levels (A, corresponding internal standard response is AIS, MA is the mean of three A/AIS). Matrix factor = (B/ BIS- MA)/MA *100%.

The stability of analytes in matrix were evaluated by the spiked QCs with six replicates. The stability of biological samples after three repeated freeze–thaw cycles at -20℃ was evaluated, as well as the stability of long-term storage at − 20℃, short- term at room temperature, automatic sample tray placement at 2–8℃.

Application to a pharmacokinetic study

The grouping, administration, sample collection, and sample pretreatment of PPD are detailed in my previous article[21]. 0.5 mL of venous blood was collected from the posterior venous plexus of rats, and 1.0 mL of blood was collected from the median vein of the forelimbs of dogs. The blood samples were placed in heparinized test tubes and centrifuged at 3000 rpm for 10 min to separate the plasma. After flushing the residual blood and contents on the surface with physiological saline, the tissue is weighed and placed in an analysis bag; Each organization was added with a corresponding volume of methanol at a concentration of 3 mL/g, homogenized, sonicated for 10 min, centrifuged at 3000 rpm for 10 min, and the supernatant was collected. Grind the rat feces sample evenly in a mortar, add methanol (fecal sample weight: methanol amount = 1:3) to prepare a homogenate, sonicate, centrifuge at 3000 rpm for 10 min, and take the supernatant. Urine and bile samples are stored directly. All samples are stored at -20 ℃ until analysis.

Results

Method validation

The typical chromatogram is shown in Fig. 2 (using rat plasma as an example), and the results indicated that endogenous substances in each biological matrix do not interfere with the determination of PPD.

The linear range of PPD in rat plasma, tissue, and fecal was 10–2000 ng/mL with an LLOQ of 10 ng/mL. The linear range of PPD in rat urine was 2.5–250 ng/mL with an LLOQ of 2.5 ng/mL. In rat bile, the linear range was 5–1000 ng/mL with an LLOQ of 5 ng/mL. The linear range for canine plasma spanned 5–2000 ng/mL with an LLOQ of 5 ng/mL. The correlation coefficient (r) exceeded 0.99 in all cases.

The accuracy and precision of the method were evaluated by testing three consecutive days with 18 QCs at three concentration levels, and the results are presented in Table 1.

The average extraction recoveries for rat plasma were 80.0%, 69.6%, and 80.7% at the respective concentrations. For fat, the average extraction recoveries were 50.5%, 61.5%, and 53.3%; for smooth muscle, they were 62.8%, 66.0%, and 65.3%. For urine, the average extraction recoveries were 80.2%, 86.4%, and 88.2%, while they were 82.9%, 86.9%, and 92.1% for fecal and 70.6%, 77.2%, and 87.0% for bile. Meanwhile, the average coefficient of variation of the matrix factors normalized by internal standards were not greater than 12.2%. The results indicate that the recoveries were stable and high, and were not affected by the matrix.

After undergoing three repeated freeze–thaw cycles at − 20℃, long-term storage at − 20℃, short- term at room temperature, automatic sample tray placement at 2–8℃, the average relative errors (RE%) between the measured value and the added value were less than 7.6%, − 12.3%, 4.1% and − 3.05%, respectively. The results indicate that the above conditions do not affect the stability of the sample.

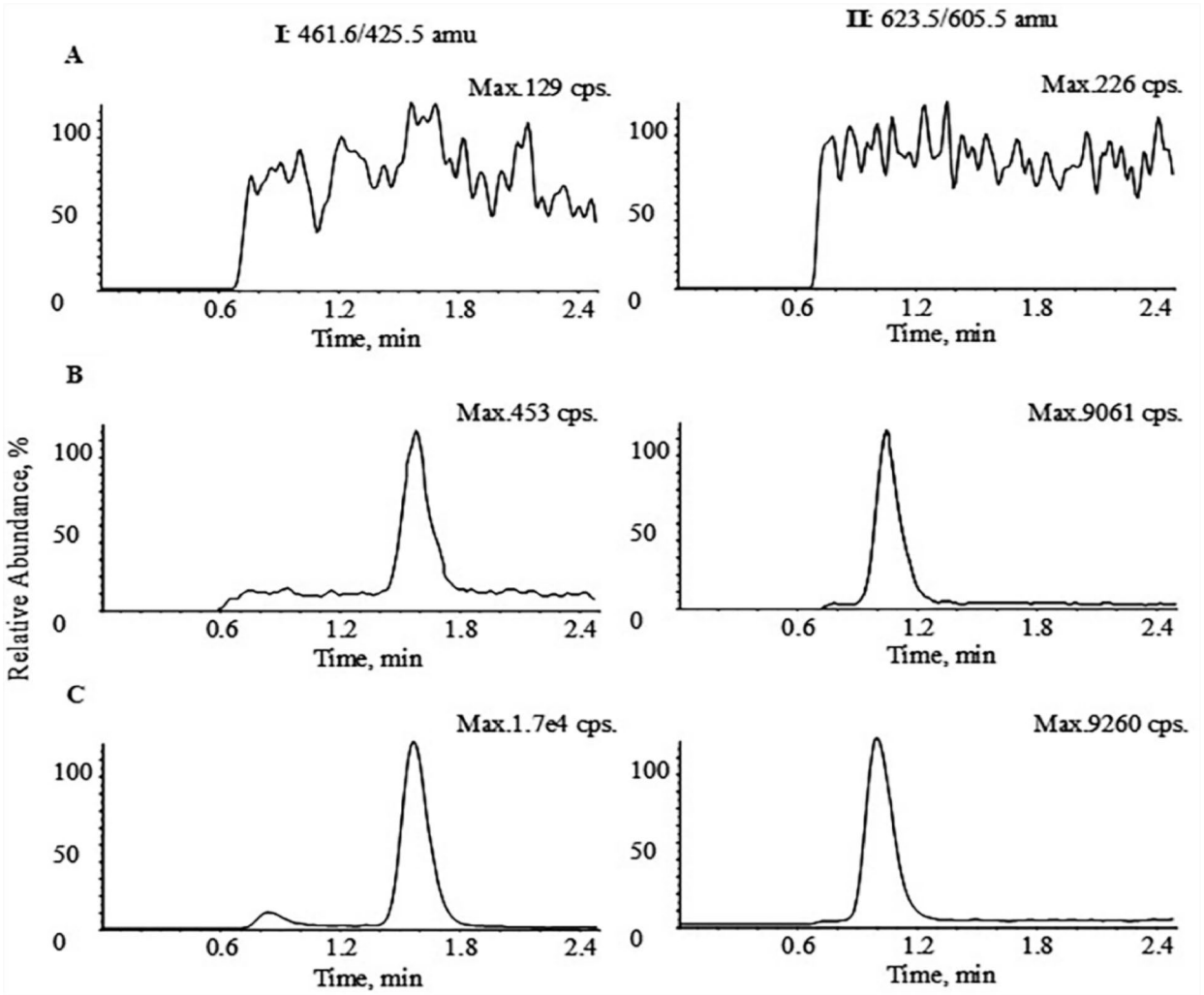

Fig. 2. MRM chromatograms of (**A**) blank plasma, (**B**) plasma spiked with PPD and ginsenoside Rh2 at the limit of quantitation (10 ng/ml) and (**C**) a plasma sample 24 h after a single oral dose of PPD (17.5 mg/kg) to a rat. Peak I, PPD; Peak II, ginsenoside Rh2.

Application to a pharmacokinetic study

After intravenous or gavage administration of PPD to rats and dogs, the mean plasma concentration–time curve is shown in Fig. 3A, B. The average absolute bioavailability of was 28.5% for rats and 11.0% for dogs. After oral gavage administration PPD to rats, we measured the distribution of the drug in 18 different tissues at different time points. After 24 h post-administration, PPD was not prone to accumulate significantly in various tissues. After oral administration of PPD in rats, the average cumulative excretion curve of PPD in urine, feces, and bile were shown in Fig. 3C, The prototype drug excretion was minimal.

Discussion

Ionization and optimization of mass spectrometry conditions

Due to PPD being a four ring three patch saponin, its molecular structure only contains C、H、O, There are no highly polar ionization centers such as N and Cl that are easily ionized. PPD's lack of ionizable centers in its structure resulted in a low mass spectrometry response. According to the structural formula, PPD contains five chemical bonds: C–C, C = C, C–H, C–O, and O–H. It is evident that compared to the other four stable chemical bonds, C-O is highly prone to breakage. The three hydroxyl groups in this compound may undergo varying degrees of intra source cleavage during ionization, making it challenging to establish a high-sensitivity mass spectrometry detection method. The primary full scan of PPD standard showed that PPD was difficult to ionize negatively. Under positive ionization conditions, only the molecular ion peak $[M+H]^+$ was observed, without the presence of $[M+NH_4]^+$ and $[M+Na]^+$ peaks, and the peak shape was not ideal. The secondary fragmentation ion scanning for $[M+H]^+$ is shown in Fig. 2A. Characteristic fragments corresponding to loss of one water molecule and two water molecules were observed at a collision voltage as low as 5 eV. Based on the structure of PPD, these fragments correspond to the removal of hydroxyl groups from the six-membered ring. Moreover, PPD underwent some intramolecular fragmentation in the electrospray ionization source itself. In

Biological matrices	Added (ng/mL)	Mean ± SD (ng/mL)	Relative error (%)	Intra-day CV (%)	Inter-day CV (%)
Rat plasma	20	19.51 ± 1.54	−2.45	7.55	10.04
	200	194.60 ± 13.22	−2.70	6.54	7.47
	1600	1657.22 ± 123.13	3.58	8.50	7.25
Rat fat	20	20.33 ± 1.10	1.63	5.17	7.53
	200	207.40 ± 9.41	3.71	4.73	1.81
	1600	1643.42 ± 61.82	2.66	3.93	1.23
Rat smooth muscle	20	20.80 ± 0.72	4.00	3.58	1.67
	200	187.70 ± 7.51	−6.17	3.82	5.54
	1600	1501.17 ± 51.62	−6.20	3.56	1.73
Rat urine	5	5.06 ± 0.31	1.15	6.01	6.68
	30	29.88 ± 2.15	−0.40	7.52	1.31
	200	212.62 ± 8.80	6.30	4.13	4.29
Rat fecal	20	22.20 ± 0.55	11.0	2.59	0.12
	200	214.37 ± 13.32	7.01	6.34	4.84
	1600	1610.89 ± 73.15	0.70	3.36	10.7
Rat bile	10	9.81 ± 0.37	−1.93	3.64	5.05
	100	97.79 ± 9.11	−2.21	9.74	2.59
	800	831.82 ± 62.78	3.97	7.93	0.25
Canine plasma	10	9.87 ± 0.70	−1.27	7.27	7.08
	200	199.48 ± 11.92	−0.27	4.95	6.04
	1600	1585.27 ± 82.38	−0.95	1.48	5.43

Table 1. Accuracy and precision of LC–MS/MS for determining PPD in different biological matrices.

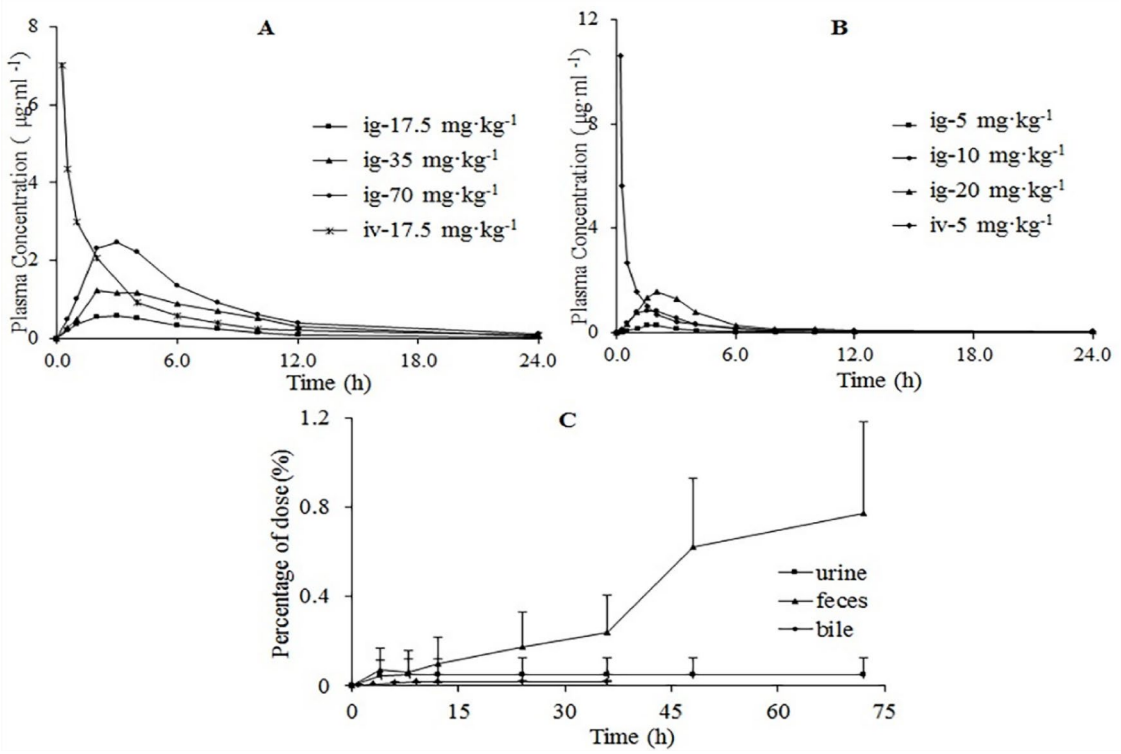

Fig. 3. Mean plasma concentration–time curve in rats (**A**) or dogs (**B**) and mean cumulative excretion curve in rats (**C**) of PPD.

order to reduce the impact of intramolecular fragmentation, the sheath gas flow was increased to 276 kPa to ensure a relatively concentrated charged ion flow, and the nebulizing gas flow and internal heating temperature were reduced to 173 kPa and 320 °C to reduce the impact of hot atomization gas on the charged ion flow. At the same time, the gas curtain flow rate was reduced to 69 kPa while ensuring the cleanliness of the ion source,

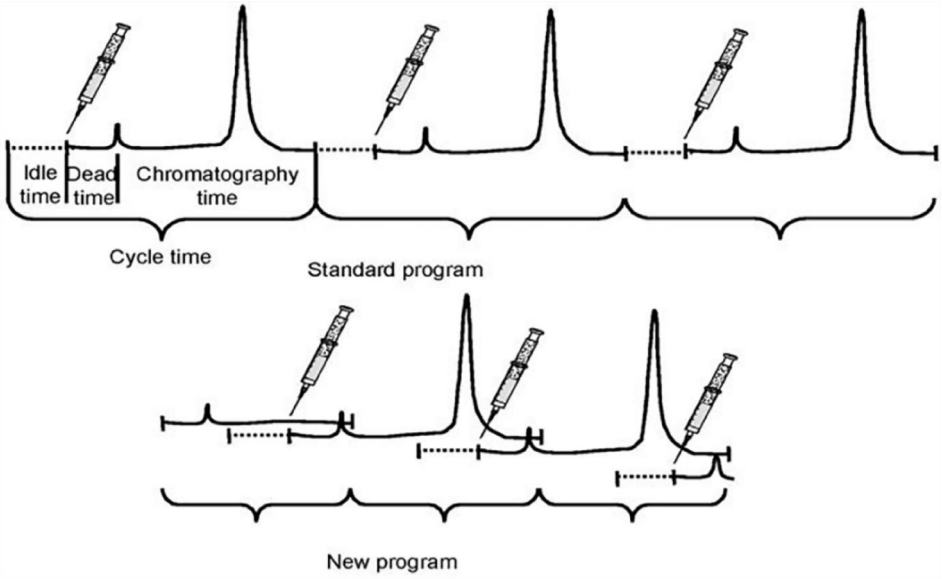

Fig. 4. The cycles involved in the operation of an autosampler controlled by the new program (programmed injection) compared with a standard program. (The original image from reference[22]).

thereby improving sensitivity. Additionally, stable pH and ion strength in the mobile phase were maintained while minimizing the presence of salt compounds.

Optimization of chromatographic conditions

During the optimization of chromatographic conditions, it was found that PPD exhibited strong retention on different reversed-phase chromatography columns. To reduce the analysis time for individual samples, a short C18 column with dimensions of 50 mm length, 2.1 mm diameter, and 3.5 μm particle size was selected, along with a high organic phase ratio of up to 90% (v/v). Regarding time optimization, we employed a novel "programmed injection" technique developed by Wang et al. in our laboratory[22]. This technique was similar to the "overlay" method used in previous liquid chromatography analysis techniques, but it further overlapped the idle time before the solvent peak and after the sample peak. We cited the original image from reference[22] to facilitate a visual comparison of how program injection saves time, as shown in Fig. 4. Finally, the analysis time was reduced from 4.2 min during method exploration to 2.4 min, resulting in significant time savings.

Selection of biological sample preprocessing methods

We tried liquid–liquid extraction, solid-phase extraction, and protein precipitation methods for the pretreatment of biological samples. Due to the presence of hydrophobic groups in the PPD structure, it was easily extractable using organic solvents for both liquid–liquid extraction and solid-phase extraction. Using ethyl acetate or a mixture of ether and dichloromethane (3:2, v/v) for liquid–liquid extraction yielded high extraction efficiencies (approximately 75%). Furthermore, better results were achieved by adding a small amount of sodium hydroxide for alkalization.

Method validation, application, and overall evaluation

Comprehensive method validation was conducted on rat plasma samples during the study, including selectivity, linearity, sensitivity, accuracy, extraction recovery, and various stability parameters. In addition, partial method validations were performed on three types of rat tissues (adipose tissue, smooth muscle, and skeletal muscle), bile, urine, fecal samples, and dog plasma samples in this study. The results were in accordance with the requirements of NMPA for bioanalytical method validation, ensuring the accuracy and reliability of our analytical measurements.

PPD exhibited a relatively low bioavailability, with complete oral absorption but substantial first-pass metabolism. The kinetic processes of PPD following intravenous administration fitted well with a three-compartment model, and after gavage administration conformed well to a two-compartment model, consistent with a linear pharmacokinetic constant ratio elimination model. There were significant interspecies differences between rats and dogs regarding PPD, whereas individual differences within the same species were minimal.

This study employed a conventional liquid–liquid extraction sample pretreatment scheme, utilizing multiple biological matrices commonly found in a single treatment protocol and liquid chromatography-tandem mass spectrometry detection parameters. The consistency of processing and detection across diverse samples eliminated the need for methodological changes, providing exceptional convenience. Although this method utilizes a relatively low-end mass spectrometry model (API4000 type) compared to literature sources[8,9,13,14,16,17], it achieves a comparable lower limit of quantification (2.5 ng/mL vs 0.5–2 ng/mL). This is primarily attributed to the utilization of a short chromatographic column separation and the innovative "programmed injection"

technology, which significantly reduces the analysis time per sample (2.4 min vs 3–5 min). In conclusion, this study introduces a groundbreaking universal LC–MS/MS method for multiple matrices (rat plasma, tissues such as adipose, smooth, and skeletal muscle, bile, urine, feces, and dog plasma), suitable for a range of tandem mass spectrometers operating at low, medium, and high levels. This method not only achieves an impressive lower limit of quantification but also offers the shortest analysis time. The method established in this study has been successfully applied to the study of the kinetic processes (absorption, distribution, metabolism, excretion) of PPD in rats and, for the first time, to the pharmacokinetic characteristics of PPD in dogs.

Conclusions

This study systematically developed and validated LC–MS/MS methods for PPD in various biological matrices, including comprehensive validation of rat plasma (selectivity, calculation curve, accuracy, precision, recovery rate, matrix effect, and stability), and partial validation of rat tissue supernatant, bile, urine, fecal supernatant, and dog plasma. A key aspect of our work is the use of a "programmed injection" technique, which significantly reduces the analysis time from 4.2 min during method exploration to 2.4 min. The method established in this study was successfully applied to the kinetic process of PPD in rats, and the pharmacokinetic characteristics of PPD in dogs were studied for the first time.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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References

1. Hasegawa, H. Proof of the mysterious efficacy of ginseng: basic and clinical trials: metabolic activation of ginsenoside: deglycosylation by intestinal bacteria and esterification with fatty acid. *J Pharmacol Sci.* **95**(2), 153–157. https://doi.org/10.1254/jphs.fmj04001x4 (2004).
2. Huang, R. et al. Studies on bioactive components of red ginseng by uhplc-ms and its effect on lipid metabolism of type 2 diabetes mellitus. *Front Nutr.* **9**, 865070. https://doi.org/10.3389/fnut.2022.865070 (2022).
3. Jin, S. et al. Detection of 13 ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, Rg3, Rh2, F1, compound K, 20 (S)-protopanaxadiol, and 20 (S)-protopanaxatriol) in human plasma and application of the analytical method to human pharmacokine. *Molecules* **24**(14), 2618. https://doi.org/10.3390/molecules24142618 (2019).
4. Cho, J. Y. et al. In vitro inhibitory effect of protopanaxadiol ginsenosides on tumor necrosis factor (TNF)-alpha roduction and its modulation by known TNF-alpha antagonists. *Planta Med.* **67**(3), 213–218. https://doi.org/10.1055/s-2001-12005 (2001).
5. Lee, J. Y. et al. Antitumor promotional effects of a novel intestinal bacterial metabolite (IH-901) derived from the protopanaxadiol-type ginsenosides in mouse skin. *Carcinogenesis* **26**(2), 359–367. https://doi.org/10.1093/carcin/bgh313 (2005) (**Epub 2004 Oct 21**).
6. Tanaka, O., Nagai, M. & Shibata, S. Chemical studies on the oriental plant drugs: The stereochemistry of protopanaxadiol, a genuine sapogenin of ginseng. *Chem Pharm Bull.* **14**(10), 1150–1156. https://doi.org/10.1248/cpb.14.1150 (1966).
7. Xu, Q. F., Fang, X. L. & Chen, D. F. Pharmacokinetics and bioavailability of ginsenoside Rb, and Rg, from Panax notoginseng in rats. *J Ethnopharmacology.* **84**(23), 187–192. https://doi.org/10.1016/s0378-8741(02)00317-3 (2003).
8. Han, M., Chen, J., Chen, S. & Wang, X. Development of a UPLC-ESI-MS/MS assay for 20(S)-protopanaxadiol and pharmacokinetic application of its two formulations in rats. *Anal Sci.* **26**(7), 749–753. https://doi.org/10.2116/analsci.26.749 (2010).
9. Li, J. Q. et al. Simultaneous determination of 20(S)-protopanaxadiol and its three metabolites in rat plasma by LC-MS/MS: application to their pharmacokinetic studies. *Biomed Chromatogr.* **32**(8), e4252. https://doi.org/10.1002/bmc.4252 (2018) (**Epub 2018 May 2**).
10. Choi, I. D. et al. Enhanced absorption study of ginsenoside compound K (20-O-β-(D-Glucopyranosyl)-20(S)-protopanaxadiol) after oral administration of fermented red ginseng extract (HYFRG™) in Healthy Korean Volunteers and Rats. *Evid Based Complement Alternat Med.* https://doi.org/10.1155/2016/3908142 (2016) (**Epub 2016 Jul 19**).
11. Shangguan, D. et al. New method for high-performance liquid chromatographic separation and fluorescence detection of ginsenosides. *J Chromatogr A.* **910**(2), 367–372. https://doi.org/10.1016/s0021-9673(00)01208-5 (2001).
12. Cui, J. F., Björkhem, I. & Eneroth, P. Gas chromatographic-mass spectrometric determination of 20(S)-protopanaxadiol and 20(S)-protopanaxatriol for study on human urinary excretion of ginsenosides after ingestion of ginseng preparations. *J Chromatogr B Biomed Sci Appl.* **689**(2), 349–355. https://doi.org/10.1016/s0378-4347(96)00304-0 (1997).
13. Zhang, D. et al. Rapid and sensitive LC-MS/MS assay for the quantitation of 20(S)-protopanaxadiol in human plasma. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **877**(5–6), 581–585. https://doi.org/10.1016/j.jchromb.2009.01.009 (2009) (**Epub 2009 Jan 14**).
14. Zhao, X. E. et al. Dual ultrasonic-assisted dispersive liquid-liquid microextraction coupled with microwave-assisted derivatization for simultaneous determination of 20(S)-protopanaxadiol and 20(S)-protopanaxatriol by ultra high performance liquid chromatography-tandem mass spectrometry. *J Chromatogr A.* **11**(1437), 49–57. https://doi.org/10.1016/j.chroma.2016.02.017 (2016) (**Epub 2016 Feb 6**).
15. Cai Xiaojun, Xu., Yuping, P. Y., Yiping, R. & Zheng, X. An UFLC-MS/MS method for quantification of panaxadiol in rat plasma and its application to a pharmacokinetic study. *Planta Med.* **79**(14), 1324–1328. https://doi.org/10.1055/s-0033-1350675 (2013) (**Epub 2013 Aug 7**).
16. Ren, H. C. et al. Sensitive determination of 20(S)-protopanaxadiol in rat plasma using HPLC-APCI-MS: Application of pharmacokinetic study in rats. *J Pharm Biomed Anal.* **48**(5), 1476–1480. https://doi.org/10.1016/j.jpba.2008.09.045 (2008) (**Epub 2008 Oct 8**).
17. Bao, Y., Wang, Q. & Tang, P. Lithium adduct as precursor ion for sensitive and rapid quantification of 20 (S)-protopanaxadiol in rat plasma by liquid chromatography/quadrupole linear ion trap mass spectrometry and application to rat pharmacokinetic study. *J Mass Spectrom.* **48**(3), 399–405. https://doi.org/10.1002/jms.3174 (2013).
18. Wang, W., Shao, Y., Ma, S., Xiangmeng, Wu. & Meng, Q. Determination of 20(S)-protopanaxadiol ocotillol type epimers in rat plasma by liquid chromatography tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci.* **1**(887–888), 19–24. https://doi.org/10.1016/j.jchromb.2011.12.032 (2012) (**Epub 2012 Jan 16**).

19. Yang, Li. et al. LC-MS/MS determination of ginsenoside compound K and its metabolite 20 (S)-protopanaxadiol in human plasma and urine: applications in a clinical study. *Bioanalysis* **11**(5), 365–380. https://doi.org/10.4155/bio-2018-0185 (2019) (**Epub 2019 Mar 15**).
20. Percie du Sert, N. et al. The ARRIVE guidelines 20: Updated guidelines for reporting animal research. *BMC Vet Res.* **16**(1), 242. https://doi.org/10.1186/s12917-020-02451-y (2020).
21. Li, P., Zhang, M., Chen, M., Liu, G., Meng*, L., Zhang*, D. Systematic studies on the kinetic process of 20(S)-protopanaxadiol in rats and dogs: absorption, distribution, metabolism and excretion. *Front. Pharmacol.* 2024: 1430780. https://doi.org/10.3389/fphar.2024.1430780.
22. Wang, Y., Zhong, D., Fawcett, J. P. & Gu, J. Autosampler programming for improved sample throughput in liquid chromatography/mass spectrometry. *Clin Chem.* **51**(11), 2216–2218. https://doi.org/10.1373/clinchem.2005.056556 (2005).

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Declarations

Competing interests
The authors declare no competing interests.

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