A Simple and Rapid Method for the Determination of Pennogenin Diglycoside in Rat Plasma by HPLC–MS: Application to the Pharmacokinetics of the Extract in Gongxuening Capsules

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Abstract

A sensitive, selective, and reproducible reversed-phase high-performance liquid chromatography method coupled with electrospray ionization-ion trap mass spectrometry (LC–ESI–ITMS) was developed for the simultaneous quantification of pennogenin diglycoside (PD) in a small volume (100 µL) of rat plasma. PD was extracted from rat plasma samples using liquid–liquid extraction with methanol, digoxin as internal standard. The chromatographic separation was performed on a reversed-phase Agilent C_{18} (250 mm × 4.6 mm i.d., 5 µm particle size) analytical column using a mobile phase of 0.1% formic acid solution–acetonitrile (75:25, v/v) at a flow-rate of 1.0 mL/min. The mass spectrometer was operated in the negative ion mode at the deprotonated-molecular ions $[M-H]^-$ of parent drug. Calibration curve in spiked plasma was linear (correlation coefficient r = 0.999) from 0.5 to 50.0 mg/mL. For the different samples with concentration of 0.50, 5.00, and 50.0 µg/mL, recoveries of PD were (86.45 ± 4.39)%, (91.40 ± 4.40)%, and (93.79 ± 3.29)%, respectively (*n* = 3). The intra-day assay relative standard deviation at 0.50, 5.00, and 50.0 mg/mL of PD were 4.29%, 5.66%, and 4.03% (*n* = 3), respectively. The inter-day assay %RSD at the previously mentioned concentrations were 5.53%, 4.99%, and 4.31% (*n* = 3), respectively. The method was successfully applied to the pharmacokinetic study of PD in rats following either intravenous administration of PD solution or oral administration of the extract in Gongxuening capsules, a famous patent Chinese botanic drug.

Introduction

The Gongxuening capsule is a famous patent Chinese botanic drug, prepared with the extract of rhizome of *Paris polyphylla* Smith *var. yunnanensis* (Franch.) Hand. –Mazz. or *Paris polyphylla* Smith *var. chinensis* (Franch.) Hara. In China, it is widely used in clinics to treat bleeding, such as excess catamenia, postpartum flooding, and uterine flooding, etc. Pennogenin diglycoside (PD)($C_{39}H_{62}O_{13}$), also named the Chonglou saponin VI in China, is the main constituent of the extract. In the Chinese Pharmacopoeia (edition 2005), PD is not less than 0.52 mg per capsule with 0.13 g extract (1). The chemical structure of PD is presented in Figure 1A (2,3).

As for the methods for the determination of PD in the two kinds of plants mentioned earlier, only high-performance liquid chromatography–UV (HPLC–UV)(4) and HPLC–evaporative light scattering detector (ELSD)(5,6) have been proposed. HPLC-UV is an official method for the determination of PD in Gongxuening capsules (1). The methods for the determination of diosgenin, which has a similar chemical structure as PD, in the plants mentioned earlier, have also been reported, such as HPLC–UV (7–9) and TLC (9), which can also offer references for the determination of PD.

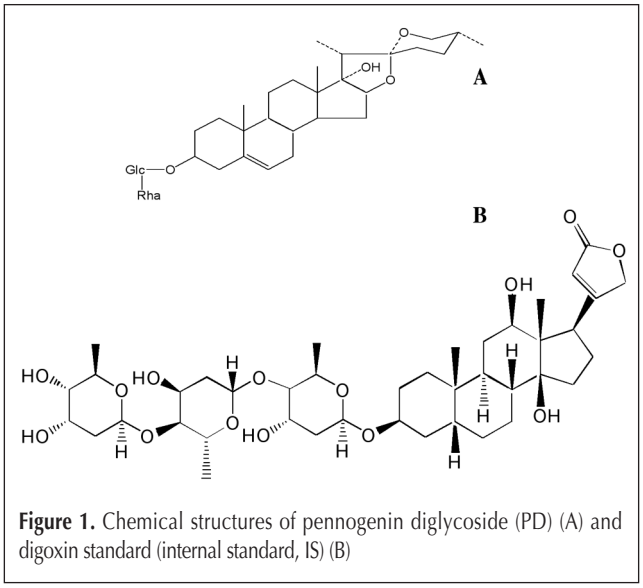

Figure 1. Chemical structures of pennogenin diglycoside (PD) (A) and digoxin standard (internal standard, IS) (B)

However, to our knowledge, there are no studies concerning the pharmacokinetics of PD following administration of single compound, herbal extract, or preparations. Only the content of Chonglou saponin in human milk was assayed with HPLC–MS after ingesting Gongxuening capsules (10). However, the paper did not report which kind of saponin was studied. Therefore, in this study, a reversed-phase HPLC assay with HPLC–MS was used for the determination of PD in rat plasma following intravenous administration of PD solution and oral administration of the extract in Gongxuening capsules in a single dose, respectively.

Experimental

Chemicals and reagent

Gongxuening capsules (Batch No. 20061008, labeled as 0.13 g extract containing 0.52 mg PD per capsule) were purchased from Yunnan Baiyao Group Co., Ltd (Kunming, China). PD standard and digoxin standard [internal standard (IS), Figure 1B] were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile was of HPLC-grade (Fisher, Waltham, MA). Distilled, deionized water was produced by a Milli-Q Reagent Water System (Millipore, Bedford, MA). All other chemicals were of analytical reagent grade.

LC-MS system

All analytical procedures were performed on an Agilent 1100 LC–MS system (Agilent Technologies Inc., Wilmington, DE), with electrospray ionization-ion trap mass spectrometry (ESI-MS) detector. The chromatographic separation was performed on an Agilent C_{18} column (4.6 × 250 mm, i.d., 5 µm) maintained at 30°C using mobile phase of 0.1% formic acid solution–acetonitrile (75:25, v/v). The flow rate was set at 1.0 mL/min. The injection volume was 20 µL.

The following optimized MS conditions were selected: Nebulizer gas (high-purity nitrogen gas, 99% purity, Beijing Gas Co., Ltd, Beijing, China.) was 20.0 psi. Dry gas flow was 6.0 L/min and dry temperature was 300°C. The voltage on the ESI interface was maintained at –3.5 kV in the negative ion mode. In the full-scan mode, the MS was operated over a range of *m/z* 100 – 1000 and the target mass was 700 *m/z*. Compound stability was 80% and trap drive level was 60%. A divert valve was used to prevent MS contamination when running LC–MS: 0–5 min, to waste; 5–15 min, to source. The selective ion monitoring was used.

Animals

Sixteen Wistar rats (8 males and 8 females), 180–220 g of body weight, were purchased from Beijing Mars Biotechnology Co., Ltd (Beijing, China). The animals were housed and cared for under a constant temperature at (22 ± 1°C) and humidity at (50 ± 10%). Food was prohibited for two days before the experiment while water was taken freely.

Intravenous and oral administration

Rats were divided into two groups and each group consisted of eight rats, four male, and four female. Twenty mg of PD was dissolved in 10.0 mL mixed solvent of 0.5 mL 1,2-propanediol, and 9.5 mL deionized water to create a PD solution. Plant extract from 50 Gongxuening capsules was dispersed into 50 mL deionized water and supersonically treated for 10 min (KQB-100, Kunshan Supersonic Instrument Co., Kunshan, China) with the final concentration of 532.8 µg/mL to create a Gongxuening dispersed solution.

For the first group, each rat was intravenously administrated the PD solution by the dose of 5 mg per kg of body weight by tail vein. For the second group, each rat was orally administrated 25 mL dispersed solution of the extract in Gongxuening capsules per kg of body weight, equal to PD 13.32 mg per kg of body weight. After administration, 0.3 mL blood samples were collected by suborbital veins for each rat at 5, 10, 15, 30, 45, 60, 75, 90, 120, 180, 240, and 300 min, respectively. The rats were gently anesthetized with ether before blood samples were collected.

Sample preparation

Each collected blood sample was immediately centrifuged at 4500 rpm for 10 min at 4°C (TGL 16C, Medical Centrifuge Co., Beijing, China). The resulting plasma (100 µL) was mixed with 10 µL IS solution (10 µg/mL) and 4-fold volume of methanol using a vortex for 3 min. The tube was centrifuged at 4500 rpm for 5 min at 4°C. The upper layer was transferred to another tube. The extraction from plasma was repeated again. The upper layers were combined and evaporated to dryness at 45°C under a gentle stream of nitrogen. The residue was reconstituted in 100 µL methanol and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant (20 µL) was injected into the LC–MS system for analysis. The same sample handling process was used for recovery and precision determinations in plasma.

Preparation of calibration standards

The standard stock solution of 100 µg/mL of PD was prepared in methanol. A series of standard working solutions with concentrations in the range of 0.50–50.0 µg/mL for PD were obtained by further dilution of the standard stock solution with methanol. All solutions were stored at 4°C. A series of 100 µL standard working solutions were evaporated to dryness at 45 °C under a gentle stream of nitrogen. The residues were reconstituted in 100 µL of blank plasma to prepare the calibration standards containing 50.0, 30.0, 15.0, 5.00, 2.00, 1.00, and 0.50 µg/mL for PD by the same method of blood sample preparation.

Validation study

Preparation of calibration curve

Linearity of calibration was tested by extraction and assayed as described previously (n = 3). Calibration curve in the concentration range of 0.50–0.50 µg/mL for PD was determined by plotting the ratio of peak area for PD to IS (A_{PD}/A_{IS}) versus concentration in plasma. Unknown concentrations of PD were determined with reference to the calibration line.

Analytical recovery

The analytical recovery of the extraction procedure for PD from rat plasma was determined by spiking 100 µL aliquots of drug-free plasma with various amounts of PD. Plasma samples were spiked with PD at 0.50, 5.00, and 50.0 µg/mL and extracted according to

the method described previously ($n = 3$). Then, 100 ng (10 µL of 10 µg/mL) of IS was added to the extracts and the sample was injected into the LC–MS. The ratio of area for PD to IS was calculated. Standard mixtures of PD and IS equivalent to the concentration in the plasma samples were directly injected into the LC–MS and the ratio of area for PD to IS was calculated. The recovery of PD was evaluated by comparing the peak area ratios of PD/IS in the plasma extracts to that of the standard mixtures.

Assay precision and accuracy

Quality control (QC) samples were prepared in the same way as calibration standards with blank plasma, and the nominal concentrations were 0.50, 5.00, and 50.0 µg/mL, respectively, corresponding to the low QC (LQC), medium QC (MQC), and high QC (HQC) levels of the calibration curve. The samples were stored at –80°C until analysis. Intra-day accuracy and precision were tested by analysis of the QC samples at different times during the same day. Inter-day accuracy and precision were determined by repeated analysis of the same samples over three consecutive days. The concentration of each sample was determined using calibration standards prepared on the same day. The percent relative standard deviation (%RSD) was used as an index of precision. Accuracy was calculated by comparing the mean experimental concentrations of assayed QC standards with their nominal values, and percent relative error (%RE) was used as the index. The equation of RE was

$$RE = (C_1 - C_2) / C_2 \times 100\%,$$

where C_1 was the mean of determined concentration, and C_2 was the nominal concentration (11).

Stability

The stability of the plasma samples was examined at room temperature for 4 h as well as stored at –80ºC for seven days. The data from the same samples of three freeze and thaw cycles from –80°C to room temperature were also analyzed without further delay. The amount of PD in the plasma samples was determined using a newly prepared calibration curve.

Assay application

The present method was used to determine concentration-time profiles of PD in rat plasma after intravenous administration of PD solution and oral administration of Gongxuening-dispersed solution, respectively. The concentrations of PD were determined by using the equation of linear regression obtained from the calibration curve.

DAS 2.0 pharmacokinetic software (provided by Chinese Pharmacology Association) was used to describe the compartment-model of the plasma concentration-time curve by compartmental analysis and calculate main pharmacokinetic parameters. The area under the concentration-time curve (AUC_{0-t}) was calculated by the trapezoidal method. Pharmacokinetic parameters were derived from the means of the individual results.

Results and Discussion

Chromatography and mass spectrum

In order to make the peaks smooth and symmetrical, each extract ion chromatogram was smoothed twice. The extract ion chromatograms (EIC) and the mass spectra (MS) of PD are shown in Figure 2. The analytes formed predominantly negative ionized molecules $[M–H]^-$ in full-scan spectra. From the MS, it could be found that the molecule with *m/z* 783 was $[M+HCOOH–H]^-$, the molecule conjugated formic acid from mobile phase. The molecule with *m/z* 737 of MS^2 was $[M–H]^-$. From the fragments of MS^3, it could be identified that the molecule with *m/z* 591 was

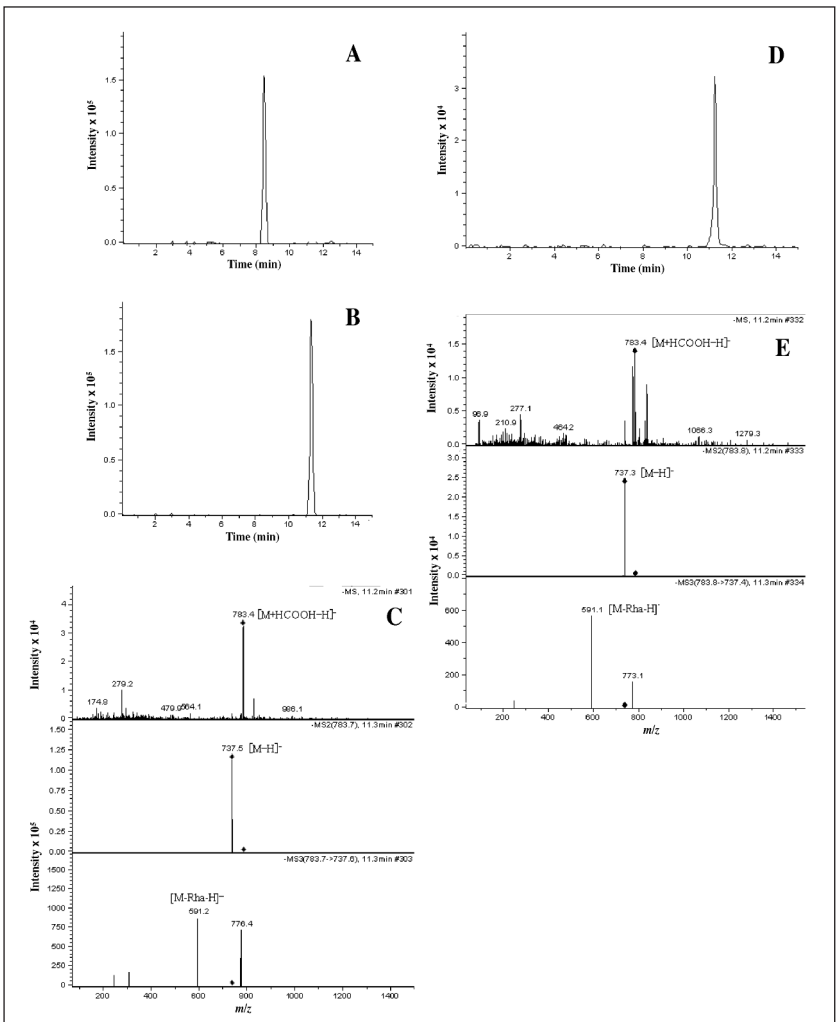

Figure 2. EIC and MS of pennogenin diglycoside (PD) in rat plasma: EIC of digoxin standard (internal standard, IS) in blank plasma (A), EIC of PD in blank plasma spiked with PD (B), MS of PD in blank plasma spiked with PD (C), EIC of PD in plasma at 5 min after single oral dose of the dispersed solution of extract in Gongxuening capsules (D), MS of PD in plasma at 5 min after single oral dose of the dispersed solution of extract in Gongxuening capsules (E).

[M–Rha–H]$^-$. Hence, it could be identified that the peak in chromatogram of Figure 2D was PD with a retention time of approximately 11.2 min. The assay was considered adequately specific for no endogenous plasma components eluted at the same retention times of the analytes of interest.

Calibration curve

The calibration curve of PD was constructed in the range 0.50–50.0 µg/mL. The regression equation of the curve was calculated as follows: $y = 0.0086x - 0.0018$ (correlation coefficient $r = 0.999$), where x was the concentration of PD (µg/mL) and y was the ratio of area for PD to IS. It showed good linear relationships between y and x.

Recovery

Recovery data are shown in Table I. For the different samples with concentration of 0.50, 5.00, and 50.0 µg/mL, recoveries of PD were (86.45 ± 4.39%), (91.40 ± 4.40%), and (93.79 ± 3.29%), respectively ($n = 3$). The mean recovery was (90.54 ± 3.74%), showing that the liquid–liquid extraction method established could extract the most of PD from plasma. Some studies have reported that the liquid–liquid extraction method, treating samples with methanol only once, could extract most of saponin (more than 90%) from blood samples (12,13), indicating that this liquid–liquid extraction method was simple, rapid, and of high recovery.

Assay precision and accuracy

Assay precision and accuracy data are shown in Table II. The intra-day assay %RSD at 0.50, 5.00, and 50.0 mg/mL of PD were 4.29%, 5.66%, and 4.03%, respectively. The inter-day assay %RSD at the above concentrations are 5.53%, 4.99%, and 4.31%, respectively. For LQC, RE was satisfactory for both intra- and inter-day assay. For MQC and HQC, REs was less than 5%.

Table I. Recovery of PD in Rat Plasma ($n = 3$)

Spiked concentration (µg/mL)	No. of duplicates (n)	Recovery (%) (mean ± SD)	RSD (%)
0.50	3	86.45 ± 4.39	5.1
5.00	3	91.40 ± 4.40	4.8
50.0	3	93.79 ± 3.29	3.5

Table II. Intra-day and Inter-day Assay Precision and Accuracy of PD in Rat Plasma ($n = 3$)

Nominal concentration (µg/mL)	Estimated concentration (µg/mL) (mean ± SD)	Precision (RSD%)	Accuracy (RE%)
Intra-day			
0.50	0.559 ± 0.024	4.29	11.8
5.00	4.77 ± 0.27	5.66	–4.6
50.0	47.89 ± 1.93	4.03	–4.2
Inter-day			
0.50	0.561 ± 0.031	5.53	12.2
5.00	4.81 ± 0.24	4.99	–3.8
50.0	47.75 ± 2.06	4.31	–4.5

Stability

Stability was expressed as a percentage of the nominal concentration. The results proved that after standing at room temperature for 4 h, going through three freeze and thaw cycles from –80ºC to room temperature, and stored at –80ºC for seven days, RSDs ($n = 3$) were 1.3%, 3.8%, and 2.7%, respectively, which meant that the samples were stable under the conditions earlier.

Application

The assay method described here was successfully used to quantitatively measure the concentrations of PD in plasma samples obtained from rats intravenously administered PD solution and orally administered Gongxuening dispersed solution, respectively.

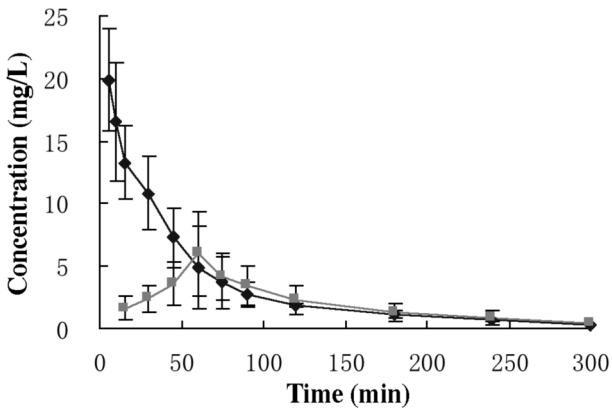

Figure 3. Rat plasma concentration-time profiles of pennogenin diglycoside (PD) following single intravenous dose of PD solution (—◆—) and single oral dose of the dispersed solution of extract in Gongxuening Capsules (—▲—), respectively ($n = 8$)

Table III. Pharmacokinetic Parameters of PD After Single Intravenous Dose of PD Solution and Oral Dose of the Dispersed Solution of Extract in Gongxuening Capsules to Rats ($n = 6$)

Parameters	IV	Oral
$t_{1/2\alpha}$ (min)	16.4 ± 8.2	43.3 ± 22.6
$t_{1/2\beta}$ (min)	66.5 ± 4.8	65.3 ± 6.9
AUC_{0-t} (mg/L × min)	999 ± 303	527 ± 164
$AUC_{0-\infty}$ (mg/L × min)	1144 ± 332	595 ± 157
K_{10} (1/min)	0.021 ± 0.003	0.013 ± 0.007
K_{12} (1/min)	0.028 ± 0.024	0.009 ± 0.008
K_{21} (1/min)	0.019 ± 0.008	0.015 ± 0.008
K_a (1/min)	–	0.029 ± 0.006
MRT_{0-t} (min)	62.1 ± 9.1	109.6 ± 7.1
$MRT_{0-\infty}$ (min)	71.2 ± 12.2	135.9 ± 16.1

The plasma concentration-time profiles of PD are shown in Figure 3. Pharmacokinetic parameters are shown in Table III. After intravenous administration of PD solution, it was rapidly eliminated and $t_{1/2\beta}$ was 66.5 min. After oral administration of Gongxuening dispersed solution, the maximum plasma concentration (C_{max}) of PD was 6.0 ± 2.1 mg/L, the time to reach C_{max} (T_{max}) was 60 min, and $t_{1/2\beta}$ 65.3 min. The ($AUC_{0\text{-}t}$) was 999.3 ± 302.7and 527.1 ± 163.5 mg/L × min for intravenous and oral administration, respectively. The oral bioavailability (BA) of PD was calculated according to the following equation:

$$BA = \frac{AUC_{0-t(oral)}\,Dose_{(iv)}}{AUC_{0-t(iv)}\,Dose_{(oral)}} \times 100\%$$

The result showed that BA was 19.80% for oral administration of Gongxuening dispersed solution compared with intravenous administration of PD solution.

Conclusion

A simple and rapid method was developed for the identification and quantification of PD in rat plasma by HPLC–MS. The method could be used for identification and quantification at the same time. The assay had good sensitivity and repeatability to fit the need of determination. The method had good selectivity with no interference from other analytes. The method established could be successfully used in pharmacokinetics of PD in Gongxuening capsules, a famous patent Chinese botanic drug.

Acknowledgements

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