Journal of Chromatography B, 878 (2010) 102–106

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

An LC-MS/MS method for determination of forsythiaside in rat plasma and application to a pharmacokinetic study

Geng-Nan Wang[a], Rui-Le Pan[a], Yong-Hong Liao[a], Ying Chen[a], Jing-Tian Tang[b], Qi Chang[a,*]

[a] Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, No. 151, Malianwa North Road, Haidian District, Beijing 100193, PR China
[b] Institute of Medicinal Physics & Engineering, Tsinghua University, Beijing 100084, PR China

ARTICLE INFO

Article history:
Received 31 August 2009
Accepted 15 November 2009
Available online 18 November 2009

Keywords:
Forsythiaside
LC-MS/MS
Pharmacokinetics
Bioavailability

ABSTRACT

A highly sensitive liquid chromatography-tandem mass spectrometric (LC-MS/MS) method was developed for the determination of forsythiaside in rat plasma using epicatechin as internal standard. The analytes were extracted by solid-phase extraction and chromatographied on a C_{18} column eluted with a gradient mobile phase of acetonitrile and water both containing 0.2% formic acid. The detection was performed by negative ion electrospray ionization in multiple reaction monitoring mode, monitoring the transitions m/z 623 → 161 and m/z 289 → 109 for forsythiaside and epicatechin, respectively. The assay was linear over the concentration ranges of 2.0–50.0 and 50.0–5000.0 ng/mL with limits of detection and quantification of 0.2 and 1.0 ng/mL, respectively. The precision was <10.8% and the accuracy was >91.9%, and extraction recovery ranged from 81.3% to 85.0%. This method was successfully applied to a pharmacokinetic study of forsythiaside in rats after intravenous (20 mg/kg) and oral (100 mg/kg) administration, and the result showed that the compound was poorly absorbed with an absolute bioavailability being approximately 0.5%.

1. Introduction

Forsythiaside, a phenylethanoid glycoside (Fig. 1), is the most abundant component of a very well-known Chinese herbal medicine Lian-Qiao, which is the fruit of *Forsythia suspense* (Thunb) Vahl. The herb has been widely used as an antipyretic, antidotal and anti-inflammatory agent in China, Japan and Korea for the treatment of various infections, especially acute upper respiratory tract complaints caused by viruses and/or bacteria infection [1,2]. Forsythiaside together with phillyrin are commonly used as chemical markers for quality control of Lian-Qiao raw material [3–5] and the derivated preparations [6,7]. The content of forsythiaside is about 4–7% in its dry green fruits named Qing-Qiao and 0.8–3% in its ripe fruits named Lao-Qiao [3–5]. Pharmacological studies demonstrated that forsythiaside possesses strong antioxidant [8,9], antibacterial [8] and antiviral [10,11] activities, and also exhibits a slow relaxation effect against norepinephrine induced contraction of rat aorta [12]. Moreover, it is reported that forsythiaside could significantly protect DNA damage caused by hydroxyl radicals [13] and inhibit protein kinase C (PKCα) with an IC_{50} value of 1.9 μM [14].

However, the pharmacokinetic characteristics and absorption profile in the gastrointestinal tract of forsythiaside are largely unknown so far, due to lack of sensitive assays. Previously documented methods for the analysis of forsythiaside in biological samples and the study of the pharmacokinetic properties in rats or dogs after intravenous (i.v.) administration were carried out using HPLC coupled with ultraviolet (UV) detection [15–18]. Such methods were not sensitive enough for oral plasma assay, with limit of detection (LOD) and quantification (LOQ) higher than 23 and 52 ng/mL, respectively, when 150 or 200 μL plasma were used. As a result, the objective of the present study was to develop a highly selective LC-MS/MS method for the quantification of forsythiaside in rat plasma with a view to understanding the pharmacokinetics and oral bioavailability of forsythiaside in rats using the established method.

2. Experimental

2.1. Chemicals and reagents

Epicatechin used as internal standard (IS) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Forsythiaside was isolated from the dried fruits of *Forsythia suspense* vahl, and its purity was found to be 97.8% by HPLC with photodiode array detection. Methanol and acetonitrile of HPLC grade were obtained from Fisher Co. Ltd. (Emerson,

* Corresponding author. Tel.: +86 10 62829823; fax: +86 10 62812595.
E-mail address: qchang@implad.ac.cn (Q. Chang).

1570-0232/$ – see front matter.
doi:10.1016/j.jchromb.2009.11.029

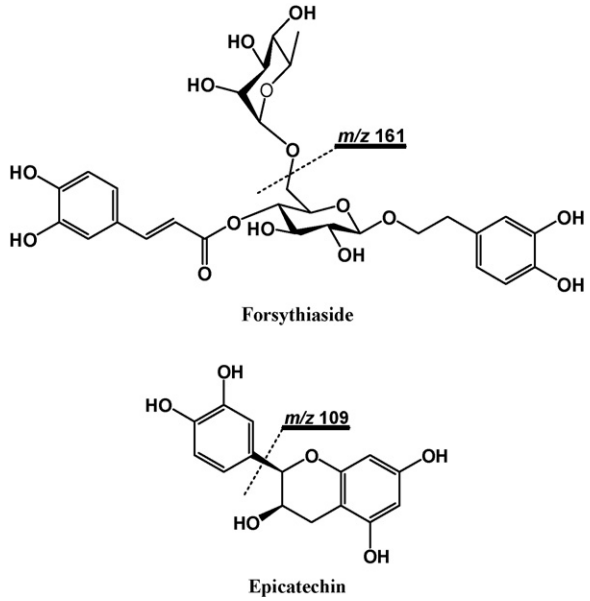

Fig. 1. Chemical structures of forsythiaside and epicatechin (IS).

IA, USA). Formic acid, hydrochloric acid and other reagents were of analytical grade and purchased from Beijing Chemical Reagent Company (Beijing, China). Milli-Q (Milford, MA, USA) water was used throughout the study.

2.2. Preparation of calibration standards and quality control samples

Stock solutions of forsythiaside and IS were prepared both in 50% methanol containing 0.1% formic acid at a concentration of 1 mg/mL and stored at −80 °C. A series of working standard solutions of forsythiaside ranging from 20.0 to 50,000.0 ng/mL and IS solution at 5 μg/mL were prepared by diluting their stock solutions with the above solvent, whose solutions were stored at −20 °C for the assay less than one week. Calibration standards were prepared using blank rat plasma (100 μL) spiked with 10 μL of forsythiaside working solutions, to yield the concentrations of 2.0, 5.0, 10.0, 20.0, 50.0, 100.0, 200.0, 500.0, 1000.0, 2000.0 and 5000.0 ng/mL, respectively. Quality control (QC) samples were prepared in the same way as the calibration samples, representing low, middle and high concentrations of forsythiaside in plasma at 7.5, 75.0, 750.0 ng/mL.

2.3. Sample preparation

A 100 μL aliquot of plasma, except for plasma (10 μL) at 5, 10 and 20 min after i.v. dosing, was vortex mixed with 20 μL of 1% formic acid, 20 μL of IS (5 μg/mL) and 1 mL of 0.1% hydrochloric acid. After centrifugation, the mixture was loaded onto an Oasis HLB cartridge (Waters, Milford, MA, USA), which was pre-conditioned with 1.2 mL of methanol followed by 1.2 mL of 0.1% hydrochloric acid. The loaded-cartridge was sequentially washed with 1.2 mL of 0.1% hydrochloric acid and 1.2 mL of water followed by vacuum dry. The analytes were then eluted with 1.2 mL of methanol. The collected eluate was concentrated to dryness by a gentle steam of nitrogen. The residue was reconstituted in 150 μL of 50% methanol in water containing 0.1% formic acid, and 50 μL of the sample was injected into LC-MS/MS system for assay.

2.4. LC-MS/MS conditions

The Agilent 1200 HPLC system (Palo Alto, CA, USA) equipped with two series 200 micro-pumps and an auto-sampler set at 4 °C was used. The chromatographic separation was achieved on a Thermo Hypersil reversed-phase C_{18} column (250 mm × 4.6 mm, 5 μm, San Jose, CA, USA), which was eluted with a gradient mobile phase of acetonitrile (A) and water (B) both containing 0.2% formic acid. The solvent A was 15% (v/v) at the beginning and linearly increased to 35% over 7 min, and then returned to 15% by 3 min. The mobile phase was delivered at a flow-rate of 1 mL/min and 40% of eluate was introduced to an Applied Biosystem 3200 Q-Trap mass spectrometer (Foster City, CA, USA) with electrospray ionization source. The detection was performed by negative ion electrospray ionization in multiple reaction monitoring mode, monitoring the transitions from molecular ion to dominant product ion m/z 623 → 161 and m/z 289 → 109 for forsythiaside and IS (Fig. 1), respectively. The optimized working parameters for mass detection was as following: curtain gas 10 psi, nebulizer gas (Gas 1) 60 psi, auxiliary gas (Gas 2) 60 psi, orifice voltage 56 V, ring voltage 60 V and turbo ion spray temperature 375 °C. All data were processed by the MassChrom software (version 1.4, Sciex).

2.5. Validation of the method

2.5.1. Linearity, sensitivity and matrix effect

The calibration curves of forsythiaside were constructed by plotting the peak-area ratios of forsythiaside/IS versus concentrations of forsythiaside in plasma. Linearity was determined using a linear least-squares regression. The LOD was defined as the plasma concentration that produced a signal-to-noise ratio (S/N) at 3. The LOQ was determined as the lowest plasma concentration that produced a S/N at 5 and could be quantified with a relative standard deviation (RSD) lower than 20% and accuracy between 80% and 120%. The matrix effect of co-extracted components in rat plasma on the ionization of forsythiaside was evaluated by comparing the peak-areas of forsythiaside and IS spiked in pre-extracted blank plasma with those of relevant standards both in reconstitution solvent at equivalent concentration.

2.5.2. Precision, accuracy and extraction recovery

Three QC samples (n = 5) were analyzed in each batch assay to determine within-run precision (RSD) and accuracy (detected concentration/nominal concentration), and analyzed in each of three different batch assays to determine between-run precision and accuracy of the method. The extraction recovery was estimated by comparing the peak-area ratio of forsythiaside to IS extracted from QC samples (n = 5) with that in solvent at equivalent concentrations.

2.5.3. Stability

The stability of forsythiaside in rat plasma was assessed using QC samples (n = 5), which were freshly prepared and immediately mixed with 20 μL of 1% formic acid followed by storing at −80 °C for two months. For stability of forsythiaside in processed samples, the prepared QC samples (n = 5) were placed in an auto-sampler at 4 °C for 24 h, and then assayed for comparison.

2.6. Application to a pharmacokinetic study

The animal experiment was approved by the Animal Ethics Committee at the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences. Male Sprague–Dawley rats (210 ± 20 g) were used and supplied by Beijing Vital Laboratory Animal Technology (Beijing, China). On the day before the experiment, a light surgery was performed. A polyethylene catheter (0.50-mm ID, 1.00-mm OD, Portex Limited, Hythe, Kent, England) was cannulated into the right jugular vein under anesthesia with an intraperitoneal dose of chloral hydrate at 350 mg/kg. After surgery, the rats were placed individually in cages and allowed to recover

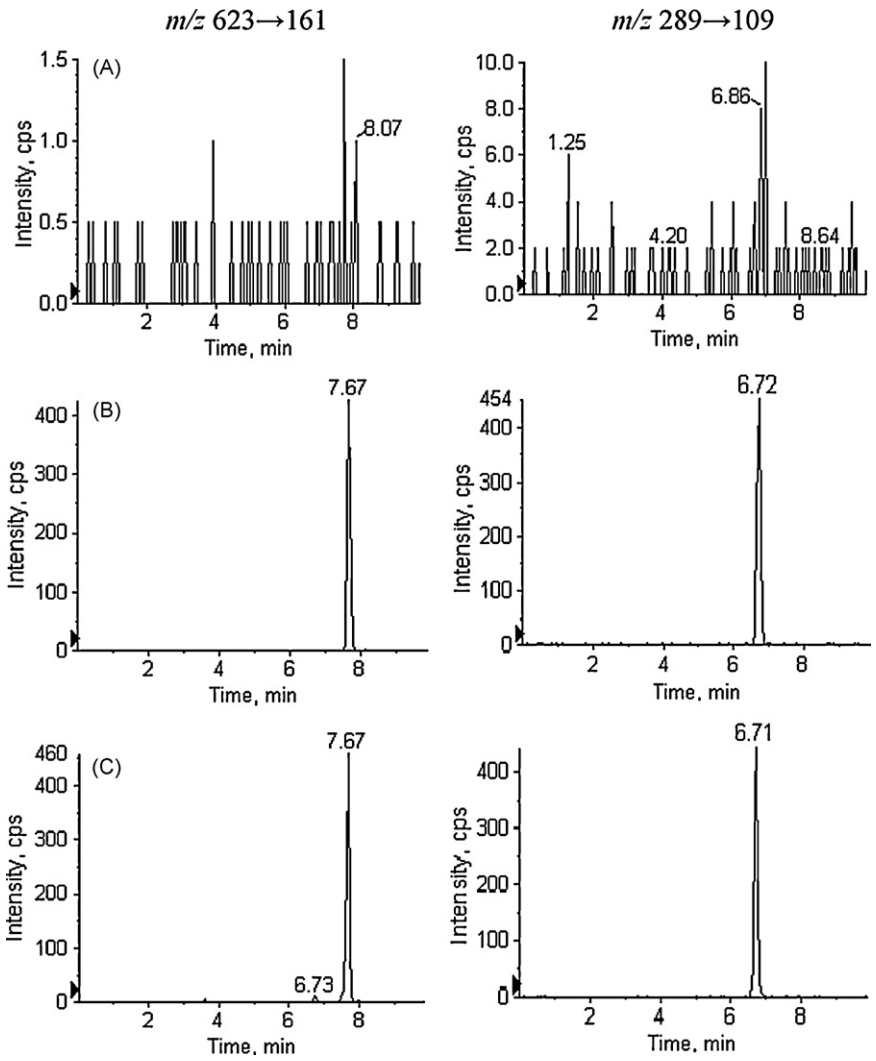

Fig. 2. Typical LC-MS/MS chromatograms of pooled blank plasma (A), blank plasma spiked with forsythiaside at concentration of 75 ng/mL and epicatechin (IS) with 20 μL of 500 ng/mL (B), and the plasma at 20 min of a rat following an single oral administration of forsythiaside at 100 mg/kg (C). Forsythiaside and epicatechin were detected at m/z 623 → 161 and 289 → 109, respectively.

for at least 12 h. The rats were fasted overnight with free access to water prior to drug administration.

Forsythiaside was freshly prepared in normal saline solution and then given to rats at a single oral dose (100 mg/kg) by gastric gavage and intravenous dose (i.v., 20 mg/kg) by rapid injection via the catheter. After i.v. administration, 0.2 mL of heparinized saline was injected into the catheter for cleaning the catheter. Rat blood samples (50–200 μL) at appropriate intervals after dosing was collected from the catheter into a heparinized centrifuge tube and centrifuged at 8000 rpm, 4 °C for 3 min for separation of plasma. The plasma samples were immediately analyzed or stored at −80 °C until assay. After each blood collection, 0.2 mL of normal saline containing 20 units of heparin was injected into the catheter to flush the catheter and prevent coagulation.

Pharmacokinetic parameters were estimated by plasma concentration versus time profiles using WinNonlin software (Pharsight Corporation, Mountain View, CA, USA, Version 2.1). Non-compartmental model was employed to calculate the following parameters, initial plasma concentration (C_0) for i.v. dose, peak plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) for oral, terminal elimination half-life ($t_{1/2,\lambda z}$), area under the plasma concentration versus time curve from zero to last sampling time (AUC_{0-t}) and infinity ($AUC_{0-\infty}$), total body clearance (CL) and volume of distribution ($V_{d,\lambda z}$). Absolute bioavailability (F) was calculated based on the $AUC_{0-\infty}$ obtained after oral and i.v. administration at the equivalent dose.

3. Results and discussion

3.1. LC-MS/MS method

Fig. 2 shows the representative LC-MS/MS chromatograms of blank plasma (A), blank plasma spiked with forsythiaside at concentration of 75.0 ng/mL (B), and the plasma at 20 min of a rat following an single oral administration of forsythiaside at 100 mg/kg (C). No interfering peak was observed in blank plasma under the assay conditions. The retention time was around 7.6 min for forsythiaside and 6.7 min for IS. Since forsythiaside is a phenolic compound, it is needed to add formic acid in the HPLC mobile phase at a concentration of 0.2% so as to overcome the peak tailing effect and further improve its detection sensitivity. The gradient elution of the mobile phase was used for narrowing the peaks of forsythiaside and IS and shortening run time of the chromatography.

Forsythiaside is a phenolic compound and susceptible to oxidation. However, it was found to be stable in acidic conditions and unstable in neutral and basic conditions (data are not showed). Therefore, the acidic solvents were used throughout the sample preparation, including collection, treatment and reconstitution

Table 1
Within-run and between-run precision and accuracy, and extraction recovery of the method for determination of forsythiaside in rat plasma.

Conc. (ng/mL)	Within-run ($n = 5$)		Between-run ($n = 5$, three runs)		Recovery (%)	
	Precision (RSD%)	Accuracy (%)	Precision (RSD%)	Accuracy (%)	Mean	RSD%
7.5	10.8	96.0	9.9	91.9	85.0	10.0
75.0	6.5	99.4	8.2	93.8	81.3	6.4
750.0	6.7	101.5	9.6	92.1	84.7	6.8

procedures. To prevent from potential degradation of forsythiaside in the blood, the fresh collected blood samples were stored on ice and then immediately centrifuged at 4 °C for separation of plasma.

Due to a wide range of plasma concentrations of forsythiaside after i.v. dosing, the calibration curve was split into two ranges of 2.0–50.0 and 50.0–5000.0 ng/mL, to avoid the undue bias to low concentrations by high concentrations. The calibration curves were $y = 0.0087x + 0.0051$ and $y = 0.0091x - 0.1349$, respectively, over the two ranges with correlation coefficient (r^2) greater than 0.992. The LOD and LOQ were 0.2 and 1.0 ng/mL, respectively, when 100 μl plasma was used for assay. Comparing the peak-areas of forsythiaside and IS spiked in pre-extracted blank plasma with those of relevant standards at equivalent concentrations, no significant increase or decrease was found for both forsythiaside and IS, indicating that the matrix effects from endogenous plasma components on the ionization of the two analytes were negligible.

The precision and accuracy data for within-run and between-run assays are shown in Table 1. The results indicate that the developed LC-MS/MS method had good reproducibility with precision less than 10.8% and excellent accuracy ranging from 91.9% to 101.5% at low (7.5 ng/mL) to high (750.0 ng/mL) concentrations. Extraction recoveries of forsythiaside were found satisfactory with average values ranging from 81.3% to 85.0% at the three QC concentrations (Table 1).

Stability study showed that the concentration of forsythiaside were not significant changed in acidified plasma stored at −80 °C for two months and in prepared plasma samples stored in a 4 °C auto-sampler for 24 h, with average remaining values ($n = 5$) of 94.1–109.2% and 93.1–99.5%, respectively. This demonstrated that forsythiaside has a good stability under the two conditions.

3.2. Application

This new developed method was applied to determine the plasma concentration of forsythiaside in rats following i.v. (20 mg/kg) and oral (100 mg/kg) administrations. The mean plasma concentration–time profiles of forsythiaside after the two doses are illustrated in Fig. 3 and its estimated pharmacokinetic parameters are presented in Table 2. It was found that forsythiaside was rapidly absorbed into the circulation system and reached its peak concentration at around 20 min after oral administration. However, its absolute bioavailability was quite low with a value being 0.5%, which value is similar with those of other phenylethanoid glycosides, echinacoside (0.83%) [19] and acteoside (0.12%) [20]. The potential hydrolysis in the gastrointestinal tract, poor permeability through the intestinal epithelial membrane and first-pass effect in the liver might be responsible for the low bioavailability of these compounds including forsythiaside.

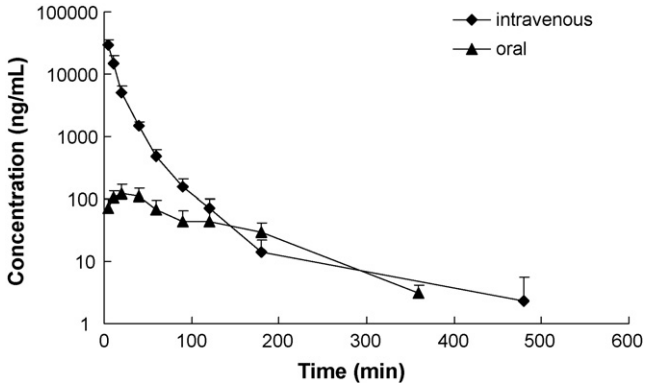

Fig. 3. Mean plasma concentration–time profiles of forsythiaside in rats following intravenous (20 mg/kg, A) and oral (100 mg/kg, B) administration. All data are expressed as mean ± SD ($n = 5$).

Table 2
Pharmacokinetic parameters of forsythiaside in rats following intravenous (i.v., 20 mg/kg) and oral (100 mg/kg) administration. All data are expressed as mean ± SD ($n = 5$).

Parameters	The route of dosing	
	i.v.	Oral
C_{max} (ng/mL)	–	122.2 ± 45.4
T_{max} (min)	0.0	20.0 ± 0.0
C_0 (μg/mL)	64.2 ± 36.1	–
$t_{1/2,\lambda z}$ (min)	76.8 ± 26.5	74.7 ± 13.3
AUC_{0-t} (μg min/mL)	570.5 ± 69.4	13.9 ± 5.2
$AUC_{0-\infty}$ (μg min/mL)	570.8 ± 69.2	14.6 ± 5.7
CL/F (mL/(min kg))	35.5 ± 4.2	39.4 ± 15.9
$V_{d,\lambda z}/F$ (L/kg)	4.0 ± 1.8	4.2 ± 1.9
F (%)	–	0.5

4. Conclusion

A sensitive and reliable LC-MS/MS method has been developed for the determination of forsythiaside in rat plasma using solid-phase extraction as sample clean-up procedure. This method showed excellent sensitivity, linearity, precision and accuracy and was successfully applied to evaluate the pharmacokinetic parameters and oral bioavailability of forsythiaside in rats.

Acknowledgement

This work was supported by the grants of International S&T Collaboration Project (20070463) and National S&T Major Project (2009ZX09301-003) both from the Ministry of Science and Technology of the People's Republic of China.

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