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Pharmacokinetics and metabolism profiles of protostemonine in rat by liquid chromatography combined with electrospray ionization tandem mass spectrometry

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ABSTRACT

A rapid and sensitive liquid chromatography/electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) method was developed and validated for determining protostemonine, a new anti-tussive agent isolated from Radix Stemonae. Separation was performed on a C_{18} column with mass detection in positive selected reaction monitoring mode at the transitions of m/z 418.2 → m/z 320.2 and m/z 416.2 → m/z 342.2 for protostemonine and internal standard, respectively. The assay showed good linearity ($r > 0.998$) over the tested concentration range with the lowest limit of quantification of 1.0 ng/ml. The intra- and inter-day precisions (RSD, %) were 2.21–9.89% and 3.99–13.19%, respectively; whereas accuracy (RR, %) ranged from 90.35% to 108.32%. The extraction recovery, stability, and matrix effect were demonstrated to be within the acceptable limits. The validated assay was further successfully applied to the pharmacokinetic studies of protostemonine in rat. Protostemonine was rapidly eliminated from plasma following single intravenous administration (2 mg/kg) with a $t_{1/2}$ of 3.06 ± 1.37 h. After oral administration (10, 20, and 50 mg/kg), protostemonine was rapidly absorbed from the gastrointestinal tract with t_{max} of approximately 1 h, and has shown dose-independent pharmacokinetic behaviors. Oral bioavailability of protostemonine was calculated to be 5.87–7.38%. Moreover, a total of 10 metabolites were structurally identified by using UHPLC-Q/TOF-MS method. The proposed metabolic pathways of protostemonine in rat involve demethylation, hydrolysis, and oxygenation. The current study provides informative data for understanding the *in vivo* disposition of protostemonine, which, in turn, help in interpreting the mechanism of its effectiveness and toxicity.

1. Introduction

Radix Stemonae has been widely used for medicinal purposes in China as anti-tussive and insecticidal agents for many years [1]. This medicinal herb, officially listed in Chinese Pharmacopoeia with the name of Baibu, comprises three species of *Stemona*, i.e., *Stemona japonica* (Blume) Miquel, *Stemona sessilifolia* (Miquel) Miquel, and *Stemona tuberosa* Loureiro, [1]. Baibu has been reported to function as an insecticide [2–4], anti-tussive [5], anti-tuberculosis, anti-bacterial, anti-helminthic [3,6], anti-fungal [7], and anti-tumor agents [8]. In the past decades, more than 80 *Stemona* alkaloids have been isolated and structurally identified from different *Stemona* species, and were widely believed to be responsible for the above-mentioned pharmacological effects [3,5–9]. Given their broad pharmacological effects, *Stemona* alkaloids have drawn increasing attention in the prevention and treatment of diseases. Protostemonine (Fig. 1), a major bioactive component isolated from the root of Stemonae, received much of our attention because of its high bioactivities. Previous study suggested that protostemonine has anti-tussive effect that could significantly antagonize citric acid-induced coughing [5]. Kaltenegger et al. [4] reported that protostemonine was shown to be highly effective in insecticidal activities with EC_{50} of 2.2 μM.

Information gained from pharmacokinetic study is essential for understanding drug safety profiles and for interpreting the mechanism of effectiveness and toxicity [10–12]. However, the information on pharmacokinetics and metabolism of *Stemona*

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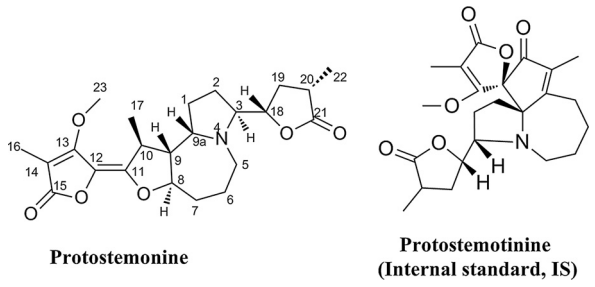

Fig. 1. Chemical structures of protostemonine and protostemotinine (internal standard, IS).

alkaloids, including protostemonine, is insufficient. Therefore, developing a simple and reliable method for pharmacokinetic study of protostemonine is necessary. Several studies have been reported to focus on the method development for determining *Stemona* alkaloids in rat plasma and on pharmacokinetic behaviors of *Stemona* alkaloids [13–15]. An LC–UV method for determination of stemoninine in rat plasma was developed and validated by Jiang et al. [13]. However, the obvious disadvantages of this method are low sensitivity and complex sample pretreatment steps, which make this method unsuitable for trace amount analysis and high throughput screening. Notably, liquid chromatography/tandem mass spectrometry (LC–MS/MS) has emerged as an alternative to the conventional LC–UV for bioanalysis, and had enable highest selectivity and sensitivity; this method is also recognized as one of the most powerful tools for quantitative and qualitative description of drug as well as its metabolites in complicated biological matrices [16–18]. Sun et al. [14–15] developed an UHPLC-Q-TOF-HDMS method to investigate the pharmacokinetics and distribution of *Stemona* alkaloids after oral administration of extract of Radix Stemonae. Although this assay allows for high sensitivity with lowest limit of quantification (LLOQ) of 0.25 ng/ml, the predominant drawback of this method is the fact that a solid phase extraction is required for sample pretreatment, which is costly and time consuming. Long run time (20 min) is another shortcoming of this method. These insufficiencies limit its application. Therefore, developing a suitable LC–MS/MS method for determining low concentrations of *Stemona* alkaloids including protostemonine in complicated biosamples is important to understand the *in vivo* disposition of *Stemona* alkaloids.

In the present study, an LC–MS/MS method coupled with methanol-mediated protein precipitation was developed and validated for determining protostemonine in rat plasma. This method was further successfully applied for the pharmacokinetic study of protostemonine in rats after intravenous and oral administrations. Furthermore, 10 metabolites of protostemonine in rat urine were identified using UHPLC-Q/TOF-MS method. The structures of these metabolites were characterized based on their MS data, MS^2 data, and retention times. Finally, the metabolic pathways of protostemonine were proposed, which suggested that hydrolysis and oxygenation were the main metabolic pathways.

2. Materials and methods

2.1. Chemicals and reagents

Protostemonine and protostemotinine (internal standard, IS) with purity of more than 98% were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals and reagents were of analytical grade and were commercially available.

2.2. Animals, drug administration, and sampling

Male Sprague-Dawley rats (200–230 g) were obtained from the Experimental Animal Center, Shanghai University of Traditional Chinese Medicine (Permit Number: SCXK (Hu) 2013–0016). The animals were kept in a breeding room at environmental temperature ($22 \pm 2\,^{\circ}C$) and 50–60% relative humidity for 7 days. A 12 h on/12 h off life cycle was employed. The rats were fed with standard laboratory food and water *ad libitum*, except for fasting 12 h prior to experiment. This study was approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (Approval Number: ACSHU-2011-G115).

For intravenous administration, five rats were given protostemonine dissolved in 0.1% acetic acid aqueous *via* tail vein at the dose of 2 mg/kg. For oral administration, fifteen rats were randomized into three groups (five rats per group), and protostemonine dissolved in 0.1% acetic acid aqueous was orally administered to rats at the doses of 10, 20, and 50 mg/kg, respectively. Approximately 150 μl blood samples were collected into heparinized tubes at predefined times. Blood samples were immediately centrifuged at $4000 \times g$ for 10 min. The resulting plasma was transferred into a clear tube and then stored at −80 °C until analysis. Protostemonine was orally administered to rats at the dose of 50 mg/kg to study the metabolism. Urine samples were collected 12 h pre-dosing and then at 0–12 and 12–24 h, respectively, and then stored at −80 °C until analysis.

2.3. Sample pretreatment

Methanol-mediated precipitation was used for the plasma pretreatment. An aliquot of 50 μl of each plasma was spiked with 50 μl of IS (100 ng/ml) and mixed for 10 s. Then, 150 μl of methanol was added and the mixture was vortexed for 30 s. After centrifuging at $19{,}000 \times g$ for 10 min, 150 μl of the supernatant was transferred into a new tube and then mixed with equal volume of double distilled water. After centrifuging at $19{,}000 \times g$ for 10 min, 10 μl of the supernatant was analyzed by LC–MS/MS.

Each urine sample (100 μl) was added with 400 μl of methanol to precipitate the proteins. After centrifuging at $19{,}000 \times g$ for 10 min, the supernatant was evaporated to dryness under a gentle nitrogen gas stream at room temperature and then re-dissolved with 200 μl of 50% methanol. The resulting solution was then centrifuged at $19{,}000 \times g$ for 10 min at 4 °C, and 10 μl of the supernatant was injected for UHPLC-Q/TOF-MS analysis.

2.4. LC–MS/MS conditions

The quantitative analysis of protostemonine was performed on an Agilent 6410B Triple Quad LC–MS/MS system (Agilent Technologies Inc., USA). Samples were separated using an ACQUITY UHPLC BEH C_{18} column (50 mm × 2.1 mm, i.d., 1.7 μm) kept at the temperature of 35 °C. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B) at the flow rate of 300 μl/min. The gradient elution program was set as follows: 0–1.5 min 10–35% B, 1.5–3 min 35–50% B, 3–4 min 50–90% B, and finally reconditioning the column with 10% B for 0.3 min. Mass conditions were as follows: gas temperature, 350 °C; gas flow, 10 l/min; nebulizer, 40 psi; capillary voltage, 4.0 kV; fragmentor voltage, 200 V; cell accelerator voltage, 0 V; and dwell time, 200 ms. Quantification transitions were set at m/z 418.2 → m/z 320.2 for protostemonine with collision energy at 38 eV, and m/z 416.2 → m/z 342.2 for IS with collision