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Full Length Research Paper

Determination of protopine in rat brain after oral administration of the extract of *Corydalis decumbentis*

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A simple and sensitive high performance liquid chromatography-ultraviolet (HPLC-UV) method was developed to determine protopine in rat brain samples after oral administration of the extract of *Corydalis decumbentis.* The extract of *C. decumbentis* was administered at a dose of 6.88 g/kg (equivalent to 159.62 mg/kg of protopine). The lower limit of quantification was 18.9 µg/ml for protopine. The calibration curve was linear over a concentration range of 21.6 to 324 µg/ml. Brain tissues samples were obtained at regular time intervals after oral administration of *C. decumbentis* extract. Protopine could be detected rapidly in rat brain after 30 min of oral administration and reach maximum concentration at about 10 h in male rat and 6 h in female rat. Furthermore, there was a great difference between male and female rats. In female rats, the maximum concentration was about twice that of male rats. This results obtained illustrate the pharmacokinetic behavior of protopine in brain tissue, and maybe helpful to explain the *C. decumbentis* bioactivity in cerebrovascular disease.

Key words: *Corydalis decumbentis*, ethanol extract, protopine.

INTRODUCTION

Corydalis decumbentis is a plant that belongs to genus *Corydalis* and is mainly distributed in Jiangxi Yujiang, China. Its *rhizoma* is a traditional Chinese medicine that promotes blood circulation and relieves pain effect, and it has been widely used for treating paralytic strokes diseases and twinge of arthritis in China for centuries (State Pharmacopoeia Commission of PRC, 2005). A lot of researches were carried to find its bioactivity components from 1980's, because of its excellence effect in cardiovascular and cerebrovascular. Until the nineties of last century, over 20 compounds have been isolated from *rhizoma C. decumbentis* and the main structure type was isoquinoline alkaloids (Guinaudeu et al., 1982; Liao et al., 1994, 1995; Zeng et al., 2005). Among them, a benzylisoquinoline alkaloid, protopine, was usually considered as a major composition used to evaluate the quality of material (Li et al., 2002; Shen et al., 2011). It is reported that protopine possesses multiple pharmacological actions, including reduces intracellular calcium (Li et al., 2005), inhibits arachidonic acid (Shen et al., 1999), suppresses thromboxane A_2 synthesis and inflammatory activity (Saeed et al., 1997).

Stroke is a brain disease. Because the blood-brain barrier hinders the delivery of many potential agents to the brain, most drugs have no effect on brain disease. So, whether the drug can penetrate the blood-brain barrier and reach the brain tissue is the first factor to be considered for a brain drug. Early publication has described the plasma pharmacokinetic study of four alkaloids, including protopine in *C. decumbentis* (Ma et al., 2009). However, it does not involve the brain tissue pharmacokinetics. Thus far, there is no documented report on the determination of protopine in brain tissue. But *C. decumbentis* as a brain drug; it is important to study the brain tissue pharmacokinetics of its main component.

In this paper, a liquid chromatography (LC) method has been developed for the quantification of protopine in rat brain tissue. This method has been successfully applied

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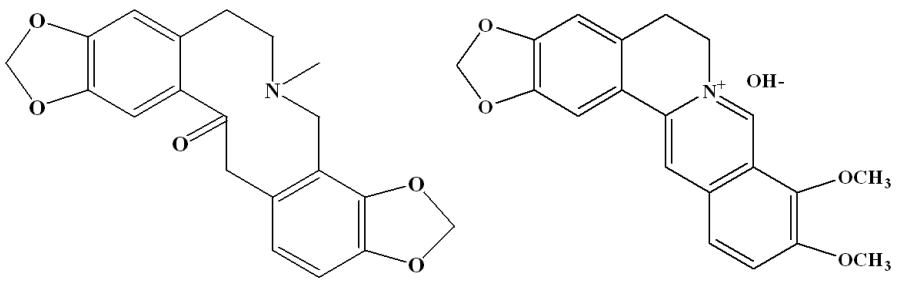

Figure 1. Structures of protopine and berberine.

to a pharmacokinetic study of protopine after oral administration of rhizoma *C. decumbentis* extract to rats.

MATERIALS AND METHODS

The crude drugs of *C. decumbentis* rhizoma were purchased from Yujiang (Jiangxi). The raw materials were identified by Professor Junqing Zhang (Hainan Medical University). The voucher specimens (No. Kanion-20100818) were deposited in the State Key Laboratory of Pharmaceutical Process New-tech for Chinese Medicine in Kanion Pharmaceutical Co. Ltd.

Acetonitrile for high performance liquid chromatography (HPLC) was obtained from Tedia Company (Fairfield, OH, USA), and protopine and berberine were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Water was purified by a Milli-Qplus water purification system (Millipore, Bedford, MA, USA) and was filtered with 0.22 μm membranes. All other chemicals were of analytical grade and were purchased from Shanghai Chemical Reagent Company (Shanghai China).

Extract preparation

Three hundred grams of dry rhizoma *Corydalis* was crushed into small pieces and transferred into a 5000 ml distillation flask. The mixture was refluxed with 2.0 L of 95% ethanol (v/v) for 2 h. The filtrates were collected and the residues were then refluxed twice in 2.0 L of 95% ethanol (v/v) for 1.5 h. Two batches of filtrate were combined. The solvent was removed at 60°C under vacuum and yield 40.8 g extract. To calculate the accurate administration dosage of protopine, the content of protopine in 95% ethanol extract was determined to be 2.32% using HPLC analysis method that modified the version of Chinese Pharmacopeia.

HPLC- UV analysis conditions

Reverse-phase high performance liquid chromatography (RP-HPLC) was used to analyze protopine. The experiment were all carried out with an Agilent 1200 liquid chromatography equipped with a quaternary solvent delivery system and an autosampler and a multiple wavelength detector (MWD) detector. A phenomenex ODS C_{18} column (250 × 4.6 mm i.d.; 5 μm) was adopted to analyze the samples. The mobile phase was optimized and it consisted of acetonitrile/TEA/acetic acid/water (25:0.6:2.25:72.15) at a flow rate of 1.0 ml/min. The detector was operated at 289 nm and the column temperature was set at 30°C, under these conditions, the retention time for protopine and berberine were 7.3 and 15.1 min, respectively.

Animal experiments and biosample collection

Male and female Sprague-Dawley rats were obtained from Experimental Animal Center of Yangzhou University (Certificate No. SCXK2007-0001) and they fasted overnight before the experiments. The experimental procedures were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Thirty six male and female rats weighed 180 to 200 g, *C. decumbentis* extract was dissolved in 0.5% polysorbate 80, and was administered to the rats with a dose of 6.88 g/kg (equivalent to 159.62 mg/kg of protopine), rats were sacrificed by cervical, and brain tissues were collected at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12 and 16 h after oral administration of the extract, then they were washed with physiological saline to remove blood. After it has been dried on filter paper, the brain tissues were weighed and homogenized in saline (w/v 1:4). The brain tissues were stored at -20°C until analysis.

Sample preparation

Protopine and berberine belong to isoquinoline alkaloids with similar structure (Figure 1) and berberine has no interference with rhizoma *C. decumbentis* extract in the aforementioned HPLC condition, so berberine was chose as an internal standard. Because protopine and berberine are readily soluble in chloroform, dichloromethane and similar organic solvents, so, dichloromethane was chosen as the extraction solvent for protopine and berberine. The brain tissues were removed from -20°C storage and 100 μl berberine (0.0696 μg/μl) was added, thawed under the ambient condition, then 8 ml dichloromethane was added to these samples for the extraction of protopine and berberine. After vortexing for 1 min and centrifuging at 3000 ×g for 10 min, the dichloromethane layer was sucked out with a pipette, and the upper layer was extracted twice, then, the combined organic phase was transferred into a clean centrifuge tube and was evaporated to dryness under a nitrogen stream in a 45°C water bath away from light. The residue was resolved in 200 μl methanol. Then, 10 μl was injected into the HPLC system. The ratio of protopine peak area over the internal standard was used for quantitative analysis.

Table 1. Intra- and inter-assay accuracy and precision of the method for determining protopine concentration in rat brain (*n*=5).

Concentration (ug/ml)	Accuracy (%)		RSD (%)	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
0.0216	98.2	97.8	4.7	6.8
0.108	101.3	99.1	5.6	7.2
0.324	99.1	102.1	3.9	5.3

Each value indicates the mean of five experiments.

Method validation

Calibration curve

The calibration standard samples were prepared by spiking the appropriate working solution into blank freshly brain tissues, and the final concentrations were 0.324, 0.216, 0.108, 0.054, 0.0324, and 0.0216 µg/µl. Then, 100 µl internal standard berberine (0.0696 µg/µl) was added to these samples. The samples were then extracted and analyzed as described in the sample preparation.

Recovery

The absolute recovery of protopine and berberine from rat brain homogenate was determined in replicate samples (n = 5). Namely, different amounts of protopine were added into the brain homogenate from ten naive rats to yield final concentrations of 0.0216, 0.108, and 0.324 µg/µl. Then, 100 µl internal standard berberine (0.0696 µg/µl) was added to these samples. The samples were then extracted and analyzed according to the aforementioned sample preparation procedures. The extraction recovery (ER) of protopine and berberine were calculated from the determined amounts against the added amounts of protopine and berberine, respectively.

Accuracy and precision

The intra- and inter-day precision was determined at three different concentrations (0.0216, 0.108, and 0.324 µg/µl). The inter-assay precision was studied using spiked samples that were analyzed at least five times per day within a 3-day period. Accuracy was calculated by comparing the averaged measured concentration to the nominal concentration, and was expressed in percentage.

Statistical analysis

The data was analyzed in Microsoft Excel. Results were expressed as mean ± standard deviation (SD).

RESULTS

Assay specificity

The specificity of the method was studied using blank brain tissue homogenate collected prior to administration. The chromatogram of a blank brain tissue homogenate was free of interfering peaks at the retention times of protopine (7.3 min) and berberine (15.1 min). The representative chromatograms of blank brain tissue homogenate sample (Figure 2A), brain tissue homogenate sample spiked with 216 µg/µl protopine, and 0.0696 µg/µl berberine hydrochloride (Figure 2B), male brain tissue homogenate sample 10 h after an administration of protopine (Figure 2C) and female brain tissue homogenate sample 8 h after an administration of protopine (Figure 2D).

Linearity and precision

The standard curve of protopine in brain sample was linear over the concentration range (0.0216 to 0.324 µg/µl) examined. The regression equation for protopine was y = 19.525x - 0.1301 (r = 0.9986), where y was the concentration of analytes in rat brain and x was the ratio of protopine peak area over the internal standard.

The intra-assay and inter-assay relative standard deviation (RSD) for every studied concentration was both less than 9.0%, indicating the precision of the method for routine purposes. Accuracy was calculated by comparing the averaged measured concentration to the nominal concentration, and was expressed in percentage. The results show that the concentration values are reproducible with an inter-assay RSD at the studied concentrations of less than 10%. Intra- and inter-day accuracy and precision data are shown in Table 1.

Recovery

The mean (±SD) recovery for protopine from brain homogenate samples was 89.3 ± 2.9%, 99.5 ± 9.2%, and 93.3 ± 6.7% at 0.0216, 0.108, and 0.324 µg/µl, respectively.

Lower limit of quantification

The lower limit of quantification for protopine was 0.0189 µg/µl with a signal-to-noise ratio (S/N) = 10 in brain tissue.

Pharmacokinetics study of protopine in male and female rats

The proposed HPLC method was applied to determine protopine concentrations in the rat brain tissue following

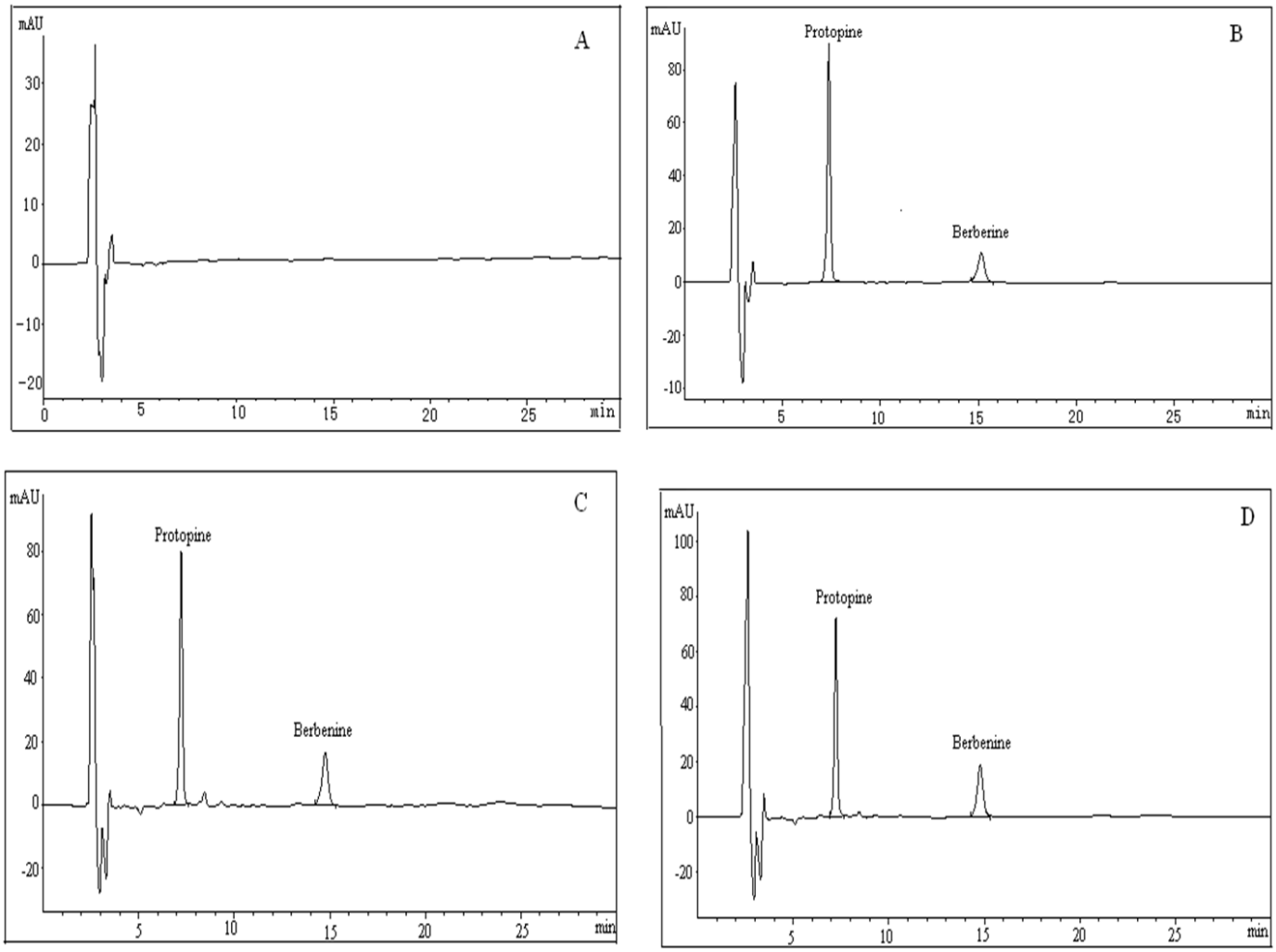

Figure 2. Typical chromatograms of the extracts from rat brain tissue homogenate. (A) Blank brain tissue homogenate sample; (B) Brain tissue homogenate sample spiked with 0.216 µg/µl protopine and 0.0696 µg/µl berberine; (C) Male brain tissue homogenate sample 10 h after oral administration of the extract (protopine 159.62 mg/kg); D. Female brain tissue homogenate sample 8 h after oral administration of the extract (protopine 159.62 mg/kg).

single intragastric (ig) dosing of *C. decumbentis* extract during a preliminary pharmacokinetic study. The kinetics of protopine in the brain of six treated rats is as shown in Figure 3. The results showed that protopine could be detected only 30 min after oral administration and achieve maximum concentration at about 10 h in male rat and 6 h in female rat. The concentration of male rat is 14.37 µg/g and that of female is 27.68 µg/g.

DISCUSSION

C. decumbentis, as it promotes blood circulation medicine, has been used clinically for a long time in China. Two products with *C. decumbentis* as the main component, Xiatianwu and Fufang Xiatianwu tablets, achieved a good therapeutic effect on squeal of stroke (Hu et al., 2005). In the present study, the brain pharmacokinetics of *C. decumbentis* extract was a meaningful work.

In the present research, a specific, sensitive, and rapid HPLC method was developed with a minor modification of previously reported assays (Ma et al., 2009; Xu et al., 2008), and this method was utilized to determine protopine in rat brain samples after oral administration. In the early pre-trial process, we also measured plasma and brain tissue concentrations of the protopine. To our surprise, we found out that protopine maximum concentration in male rat brain tissue can reach 15.6 µg/g, but the maximum plasma concentration is only up to 3.23 µg/ml. The protopine concentration in brain tissue is about five times that of plasma. So, the pharmacokinetics of protopine in brain tissue caused us great interest. Through this research, we found out that protopine can rapidly enter the central nervous system only after 10 min of its administration, and remains like

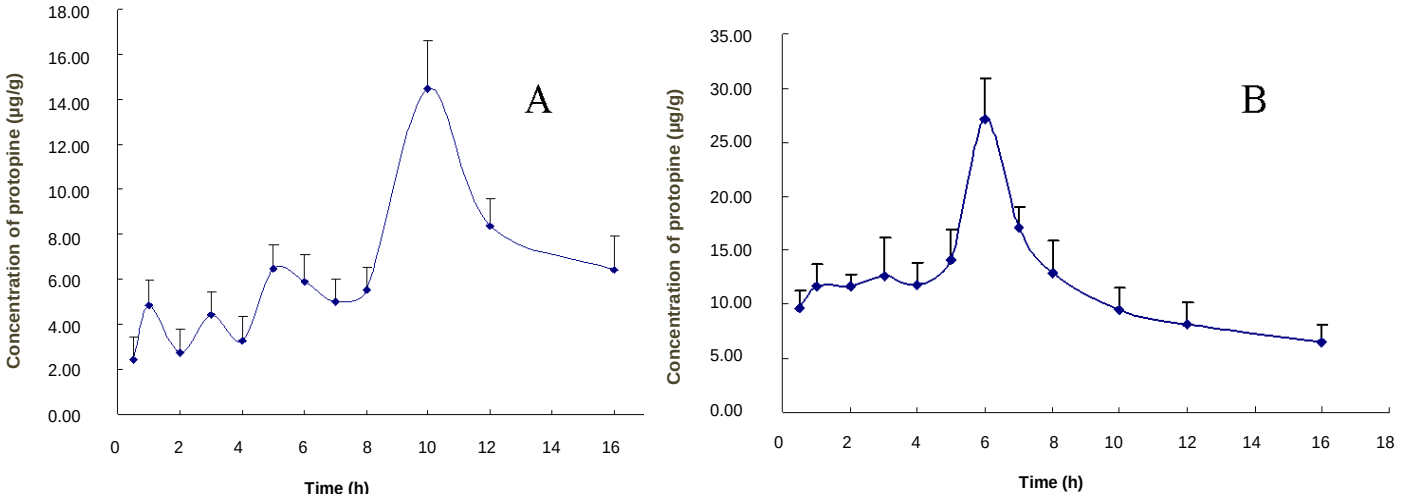

Figure 3. Kinetics of protopine in rat brain after oral administration extract (protopine 159.62mg/kg: (A) Kinetics of protopine in male rats brain tissues (3 males at each time point), (B) Kinetics of protopine in females rats brain tissues (3 females at each time point).

that for over 16 h, though the maximum concentration of female rats' brain is about twice of that of males. Therefore, after oral administration, *C. decumbentis* extract have a rapid and durable onset of action.

Conclusively, based on the result reported in this paper, it is confirmed that protopine can be quickly transported from the gastrointestinal to the brain. This result illustrates that protopine maybe a promising bioactivity component in *C. decumbentis* extract. Furthermore, the result may also provide more scientific information for further understanding of the clinical use of the herb.

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