Determination of paeoniflorin in rat hippocampus by high-performance liquid chromatography after intravenous administration of *Paeoniae* Radix extract

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Abstract

A rapid and simple high-performance liquid chromatographic (HPLC) assay for the determination of paeoniflorin in rat hippocampus was developed in this study. The chromatographic analysis was carried out using reversed-phase isocratic elution with a Zorbax SB-C$_{18}$ column, a mobile phase of methanol–water (32:68, v/v), and detection by ultraviolet (UV) absorption at 233 nm. The lower limits of quantitation (LLQ) were 1 μg/ml for paeoniflorin. The calibration curve for paeoniflorin was linear ($r = 0.9999$) over the concentration range of 1–50 μg/ml. The coefficients of variation of intra- and inter-day assays were 7.00, 0.58, 1.46% and 5.48, 1.79, 1.70% at concentrations of 1, 10, 50 μg/ml, respectively. The recoveries of paeoniflorin from rat hippocampus were 98.28 ± 2.14, 98.96 ± 1.48, and 95.34 ± 0.92% at concentrations of 1, 10 and 50 μg/ml, respectively. Stability studies showed that paeoniflorin was stable at temperatures of 2–8 °C in methanol for at least 20 days. The method was applied to determine the time course of paeoniflorin in rat hippocampus, following the administration of a 60 mg/kg i.v. dose of paeoniflorin in *Paeoniae* Radix extract to a male Wistar rat.
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1. Introduction

Paeoniae alba radix (Red Peony Root; Chishao), the dried root of *Paeonia lactiflora Pall.* or *Paeonia veitchii Lynch*, is one of the Chinese traditional crude drugs. It has long been used as a component of traditional Chinese prescription to treat certain types of dementia. Paeoniflorin (structure shown in Fig. 1), a characteristic monoterpene glucoside isolated from the root of P. *lactiflora* in 1963 [1], is one of the bioactive components in *Paeoniae* Radix. It has been reported that paeoniflorin exhibits many pharmacological effects such as anti-flammatory, anti-allergic effects [2], anti-hyperglycemic effects [3], anti-thrombosis effects [4], neuromuscular blocking [5–9], stimulating the release of noradrenaline [10], and enhancing glucose uptake [11].

Moreover, many studies indicated cognition-enhancing effects of *Paeoniae* Radix extract and/or paeoniflorin [12–18]. In brief, it was reported that the oral administration of the aqueous extract of peony root and paeoniflorin dose-dependentiy ameliorates spatial cognitive impairment caused by scopolamine in rats [12,13] via reversing muscarinic M$_1$-receptor antagonist (scopolamine)-induced inhibition of long-term potentiation (LTP), a prolonged form of synaptic plasticity in the hippocampal region [14]. Paeoniflorin also improved the learning impairment of rats with unilateral lesions of the nucleus basalis magnocellaris in a radial maze task [15] and learning deficit of aged rats in an operant brightness discrimination task [16].

Previous studies indicate that the hippocampus plays an important role in spatial memory [19,20]. It has been assumed that LTP is the synaptic basis of learning and memory [21,22]. It was reported that stimulation of muscarinic acetylcholine receptor facilitated the induction of LTP in the CA1 area of the hippocampal slice [23,24] and that blockage

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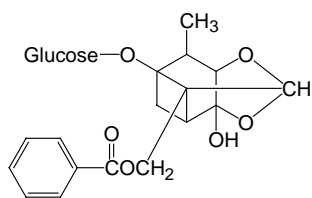*E-mail address:* pharm@mail.tsinghua.edu.cn (L. Du).

Fig. 1. Chemical structures of paeoniflorin.

of the receptor by scopolamine prevented the LTP induction [25–27].

With the increasing significance of a potential beneficial role of paeoniflorin in cognition behavior, there is a growing demand for research on its absorption, distribution and excretion in hippocampus. Furthermore, *Paeoniae* Radix extract has been administrated by the form of injections in clinical use. To ensure that the effect of *Paeoniae* Radix extract and/or paeoniflorin on cognition is a direct or indirect action, it requires, firstly, to confirm their exist in hippocampus. In the present study, therefore, we investigated the time course of paeoniflorin in rat hippocampus after intravenous administration of *Paeoniae* Radix extract. Though a few HPLC–UV methods were reported to determine paeoniflorin in crude drugs containing *Paeoniae* Radix [28,29] and in animals' plasma after oral administration [30,31], little work of paeoniflorin on cerebral nucleus has been carried out due to the lack of methodologies that meet the requirements of sensitivity and specificity for paeoniflorin determination in hippocampus. In the present study, we successfully developed a rapid and simple HPLC method to determine paeoniflorin in rat hippocampus.

2. Experimental

2.1. Chemicals and reagents

The reference standard of paeoniflorin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The internal standard, pentoxifylline, was purchased from Sigma (St. Louis, MO, USA). Acetonitrile and methanol (HPLC grade) were obtained from Fisher Scientific (New Jersey, USA). Double-distilled water was used for all preparations. *Paeoniae* Radix extract, containing 50.0% of paeoniflorin, was kindly offered by phytochemistry group of Lab of Pharmaceutical Science, Department of Biological Sciences and Biotechnology (Tsinghua University, Beijing).

2.2. Instrumentation and chromatographic conditions

The HPLC system consisted of a Waters 600 liquid chromatograph pump (Waters Co., USA), Waters 600 Controller (Waters Co., USA), 7725i injector, and a 2487 UV-Vis double-wavelength detector (Waters, USA). A Zorbax SB-C$_{18}$ reversed-phase column, 150 mm × 4.6 mm i.d.,

5 μm (Agilent, USA) was used. The signals from detector were collected and analyzed with a computer equipped with an AGE Chromstation 2000 Chromatography Manager. The mobile phase was water–methanol (32:68, v/v), filtered through a 0.45 μm Millipore filter and degassed prior to use. The flow-rate was 1.0 ml/min. The injection volume was 10 μl. Detection was performed at a wavelength of 233 nm under a constant temperature (25 ± 1 °C).

2.3. Animals

Male Wistar rats (180–230 g) were obtained from the Laboratory Animal Institute of Chinese Academy of Medical Science (Beijing, China). Animals were kept in an environmentally controlled breeding room (temperature: 20 ± 2 °C, humidity: 60 ± 5%, 12 h dark/light cycle) for 1 week before the start of the experiments. They were fed standard laboratory chow with water ad libitum and fasted overnight before the experiments. Experimental animals were maintained in accordance with internationally accepted principles for laboratory animal use.

2.4. Preparation of hippocampus samples

For the determination of paeoniflorin in hippocampus, weighted tissue samples were thawed and then homogenized in precooled water (at the ratio of 1:2, g/ml). The homogenized solution was then mixed with 2.5 ml acetonitrile by vortex for 1 min and ultrasonic for 20 min at room temperature. The denatured protein precipitate was separated by centrifugation at 1500 × g for 15 min at room temperature. The supernatant was evaporated at room temperature and then dissolved in 0.2 ml of methanol containing 50 μg/ml internal standard, pentoxifylline. A 10 μl volume of this sample solution was injected onto HPLC for analysis. The same sample handling process was used for recovery determinations in hippocampus.

2.5. Standard curve preparation

The reference substance was accurately weighted and dissolved in methanol containing 50 μg/ml internal standard, pentoxifylline, to obtain five different concentrations standard solutions in the range of 1–50 μg/ml. The content of paeoniflorin in the test samples were calculated using the regression parameters obtained from the standard curve. Calibration standards were included in every analytical batch of samples.

2.6. Application

Under anesthesia with 10% ethyl carbamate (i.p. 10 mg/kg), experimental rats were intravenously administered with *Paeoniae* Radix extracts (at a dose of 60 mg/kg of paeoniflorin) by femoral vein and were killed by snipping abdominal aorta to exsanguinate according to the specific schedule

(at times of 0, 5, 10, 15, 30, 60, 90, 120 and 240 min after dosing). Weight-matched normal rats were served as the normal control. The brain was quickly removed from the skull and placed on a glass plate over ice. The bilateral hippocampus was carefully isolated. As subsequent parts were removed, they were frozen immediately in liquid nitrogen. The parts were weighed (mean weights: 120 mg) and stored at −20 °C until assayed. Data from these samples were used to construct pharmacokinetic profiles by plotting drug concentration versus time.

3. Results

3.1. Specificity and selectivity

Paeoniflorin gave a well-defined peak with the chromatographic system used. Fig. 2 display typical chromatograms resulting from HPLC analysis of the ACN precipitated rat hippocampal tissues. Blank rat hippocampus does not demonstrate any interference peaks from endogenous components (Fig. 2a). The mixture of paeoniflorin and internal standard, pentoxifylline, in methanol solution is well separated from one to another with retention times of paeoniflorin (about 7 min), pentoxifylline (about 15 min)(Fig. 2b). The rat hippocampus samples spiked with paeoniflorin and pentoxifylline standards show similar results (Fig. 2c),

paeoniflorin and pentoxifylline are separated well from potentially interfering endogenous hippocampus compounds under the current optimal chromatographic conditions (Fig. 2a–d).

3.2. Lower limit of quantitation

The lower limit of quantification (LLQ) was determined during the evaluation of the linear range of calibration curve. LLQ was defined as the concentration of the lowest standard samples producing an assayed concentration within 10% of the theoretical value (i.e. accuracy between 90 and 110%) and yielding a precision of less than 10% for both intra- and inter-day evaluation. The LLQ of paeoniflorin was determined in methanol solution. LLQ of paeoniflorin is 1 µg/ml for methanol samples.

3.3. Linearity of calibration curve

The linearity of calibration curve was evaluated by analysis of peak area ratios (paeoniflorin/pentoxifylline) to paeoniflorin concentrations in methanol. The linearity for paeoniflorin was tested over a concentration range of 1–50 µg/ml in methanol. The typical equation of calibration curves was: $y = 3.385 \times 10^{-2}x - 1.260 \times 10^{-4}$, $r = 0.9999$ (y is the concentration of paeoniflorin; x the peak area ratio of paeoniflorin to pentoxifylline).
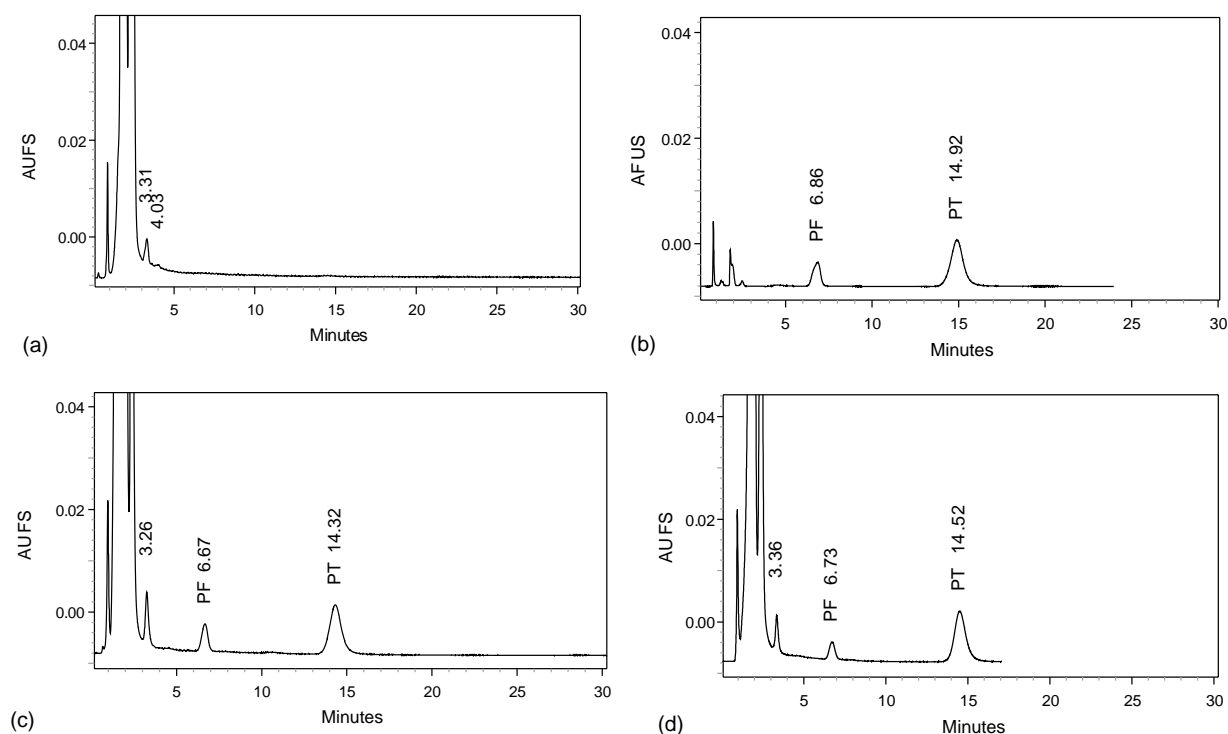

Fig. 2. (a) Chromatograms of blank hippocampus. (b) Chromatograms of methanol containing paeoniflorin (10 µg/ml) and pentoxifylline (50 µg/ml) (PF, paeoniflorin; PT, pentoxifylline). (c) Chromatograms of blank hippocampus spiked with paeoniflorin (10 µg/ml) and pentoxifylline (50 µg/ml) (PF, paeoniflorin; PT, pentoxifylline). (d) Chromatograms of rat hippocampus sample obtained 5 min after intravenous administration of *Paeoniae* Radix extract (PF, paeoniflorin; PT, pentoxifylline).

Table 1
Validation of the intra-day assay[a]

Spiked concentration (μg/ml)	Measured concentration (μg/ml)	Accuracy (%)	CV (%)
1	0.92 ± 0.06	92.11	7.00
10	9.95 ± 0.06	99.54	0.58
50	49.74 ± 0.72	99.48	1.46

[a] Each value represents the mean ± S.D. ($n = 3$).

Table 2
Validation of the inter-day assay[a]

Spiked concentration (μg/ml)	Measured concentration (μg/ml)	Accuracy (%)	CV (%)
1	0.91 ± 0.05	91.39	5.48
10	10.02 ± 0.18	100.19	1.79
50	49.90 ± 0.85	99.80	1.70

[a] Each value represents the mean ± S.D. ($n = 3$).

3.4. Precision and accuracy

The assay was validated by intra- and inter-day accuracy and precision quantifying paeoniflorin. Accuracy was determined by comparing the calculated concentration using calibration curves to known concentrations. Intra- and inter-day variability were determined for the concentration of 1, 10 and 50 μg/ml ($n = 3$). The results were summarized in Tables 1 and 2, respectively.

3.5. Recovery

The blank hippocampus samples were homogenized in precooled water (at the ratio of 1 to 2, g: ml). The homogenized solutions were spiked with three different concentrations (1, 10 and 50 μg/ml) of paeoniflorin. The following process was as same as that used for preparation of hippocampus samples (see Section 2.4). The recovery of paeoniflorin was established by comparing peak area ratios (paeoniflorin/pentoxifylline) of standards in prepared samples to those of standards in methanol. The recoveries were 98.28 ± 2.14, 98.96 ± 1.48, and 95.34 ± 0.92% at concentrations of 1, 10 and 50 μg/ml ($n = 3$), respectively (Table 3).

3.6. Stability

Paeoniflotin and petoxifylline were stable in methanol for at least 20 days when stored at 2–8 °C, and no evidence
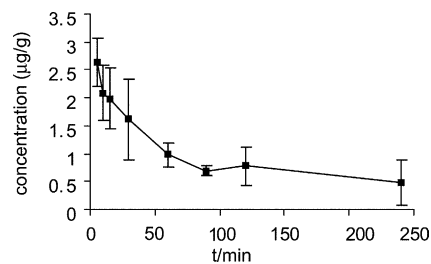

Fig. 3. Hippocampus concentration–time curve of paeoniflorin in rats brain after i.v. administration of *Paeoniae* Radix extract (at a dose containing 60 mg/kg paeoniflorin). Each point and bar represents the mean ± S.D. ($n = 5$).

showed that they arise interaction. The CV% of peak area ratios (paeoniflorin/pentoxifylline) was less than 2.5% over the concentration range of 1–50 μg/ml. Paeoniflorin in hippocampus was also stable for at least 2 months when stored at −20 °C. Prepared samples in methanol containing internal standard (50 μg/ml) were stable for at least 8 h at room temperature. Moreover, samples precipitated by acetonitrile and evaporated were stable for at least 1 month.

3.7. Application of assay in rat hippocampus

The described analytical method was used to analyze hippocampus samples following the administration of *Paeoniae* Radix extract (60 mg/kg i.v.) to a rat. The concentration of paeoniflorin in hippocampus over 2 h is plotted in Fig. 3. Using this HPLC assay, paeoniflorin was quantified in rat hippocampus.

4. Discussion

Though many previous studies have demonstrated that the hippocampus plays an important role in learning and memory and that paeoniflorin exhibits cognition-enhancing effects and ameliorates impairment of hippocampus due to various reasons including scopolamine-induced ones, studies on the distribution of paeoniflorin in rat hippocampus were not reported until now. The aim of the present study was to develop a method for paeonoflorin determination, which was used to clarify the dynamics basis underlying the ameliorative effect of paeoniflorin in the hippocampus region related to learning and memory. In this experiment, we

Table 3
Recovery of the paeoniflorin assay[a]

Spiked concentration (μg/ml)	Peak area ratio		Recovery (%)	CV (%)
	Untreated	Treated		
1	0.2978 ± 0.0017	0.2935 ± 0.0058	98.28 ± 2.14	2.17
10	2.5049 ± 0.0116	2.4786 ± 0.0275	98.96 ± 1.48	1.49
50	14.7896 ± 0.1593	14.0996 ± 0.0901	95.34 ± 0.92	0.97

[a] Each value represents the mean ± S.D. ($n = 3$).

determined the paeoniflorin and plotted the time course profile of paeoniflorin in rat hippocampus using a HPLC method.

An important finding in this study is that paeoniflorin can quickly penetrate through blood brain barrier (BBB) to reach hippocampus, which supports the pharmacological effect of paeoniflorin on hippocampus. The results revealed that paeoniflorin could quickly distribute to hippocampus after intravenous administration of *Paeoniae* Radix extract, which suggest that paeoniflorin may be directly affect certain region of hippocampus and that it may have potential ameliorative effects on some disorder such as learning and memory impairment caused by pathological agents.

In order to minimize the time used for sample preparation and to improve the recovery, the hippocampal tissues were prepared with a simple protein precipitation step. And a precolumn (10 mm × 4.6 mm i.d., 5 μm) was used to minimized the strain on the analytical columns due to incomplete precipitation of macromolecules. After different chromatographic conditions including different pH phosphate buffer solution were evaluated, a water–methanol (32:68, v/v) was found to be the optimal mobile phase.

A wavelength of 231 ± 2 nm was chosen to achieve a high selectivity against endogenous compounds in the chromatograms according to the absorption spectrum of paeoniflorin. LLQ were 1 μg/ml for paeoniflorin. The coefficients of variation of intra- and inter-day assays were 1.46–7.00 and 1.70–5.48% over the concentration range of 1–50 μg/ml, respectively. Moreover, a high recovery of paeoniflorin from rat hippocampus was achieved over the concentration range of 1–50 μg/ml, respectively. Stability studies showed that paeoniflorin in methanol and hippocampus samples was stable.

In summary, the purpose of the present study was to develop a rapid and efficient HPLC analytical method for measuring the paeoniflorin in rat hippocampus. Since this method was proven to be a good linearity, selectivity, precision and accuracy analytical method for the determination of paeoniflorin in rat hippocampus, it can be used for the biopharmaceutical study of traditional Chinese formulations containing *Paeoniae* Radix.

Acknowledgements

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