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Chinese Herbal Medicines (CHM)

ISSN 1674-6384

Journal homepage: www.tiprpress.com E-mail: chm@tiprpress.com**Original article****Determination of Protopine in Rat Brain Tissues by RRLC-ESI/Q-TOF-MS Method**Pei-xiang Wang^{1, 2}, Yong-hui Li^{1, 2}, Yan-jing Li^{1, 2}, Ting Geng^{1, 2}, Ming-li Li^{1, 2}, Gang Ding^{1, 2}, Zhen-zhong Wang^{1, 2}, Wei Xiao^{1, 2*}

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ARTICLE INFO*Article history*

Received: July 4, 2013

Revised: October 15, 2013

Accepted: January 22, 2014

Available online:

April 30, 2014

DOI:

10.1016/S1674-6384(14)60019-8

ABSTRACT

Objective To analyse the quantification of protopine from *Corydalis Decumbentis Rhizoma* (CDR) extract in brain tissues of rats. **Methods** A rapid, sensitive, and accurate analytical method based on rapid resolution liquid chromatography (RRLC) coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry (Q-TOF-MS) was developed for the quantification of protopine in brain of rats. A simple liquid-liquid extraction process was employed for the sample preparation. Chromatographic separation was achieved using 1.8 μm porous particle columns. **Results** The calibration curve of protopine was linear in the range of 12–897 ng/mL. The relative standard deviations of intra- and inter-day precision values were less than 10%. The extraction recoveries were 96.4%, 99.6%, and 98.5%, for protopine at the concentration of 598.0, 119.6, and 12.0 ng/mL, respectively, and internal standard (1.27 $\mu\text{g/mL}$) was (98.60 $\pm$ 0.02)%. **Conclusion** The validated method is successfully applied for the determination of protopine in brain tissue of rats after administration of CDR extract.

*Key words**Corydalis Decumbentis Rhizoma*; protopine; RRLC-ESI/ Q-TOF-MS; tissue distribution

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1. Introduction

Corydalis Decumbentis Rhizoma (CDR), known as *Xiatianwu* in China, is one of the commonly used Chinese materia medica (CMM) to prevent and treat diseases for thousands of years. In the past decades, over 20 compounds have been isolated from CDR. The main structure type is isoquinoline alkaloid, among which protopine is the major component (Zeng et al, 2005; Liao et al, 1994; 1995). Modern pharmacological studies had shown that protopine exhibited a broad spectrum of biological activities, such as anti-inflammatory, anti-arrhythmic, antiplatelet aggregation, anti-depressive, and anticancer effects on human hormone-

refractory prostate cancer cells (Bae et al, 2012; Song et al, 2000; Chen et al, 2001; 2012; Xu et al, 2006). Moreover, the recent studies indicated that protopine had anti-cholinesterase and anti-amnesic activities, which may ultimately hold the significant therapeutic value in alleviating certain memory impairments observed in dementia (Kim et al, 1999).

To support the results above for protopine, a sensitive analytical method to estimate the concentration of protopine in plasma/brain tissue is required. Up to now, several conventional methods, such as GC-MS, LC, and LC-MS, have been successfully established for the qualitative and quantitative analyses of protopine in raw materials or pharmaceutical preparations (Pual and Maurer, 2003;

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Fund: National Science and Technology Major Project "Creation of Major New Drugs" from China (2011ZX09401-097)

Rakotondramasy-Rabesiaka et al, 2010; Chen et al, 2009; Luo et al, 2006; Zhang et al, 2011). In addition, protopine had been determined in rat plasma using LC-MS after ig administration of CMM (Ma et al, 2009), and the tissue distribution of protopine has been investigated by HPLC (Dou et al, 2012; Li et al, 2012). However, to the best of our knowledge, no strategy had been presented for the determination of protopine in brain tissues of rats by rapid resolution liquid chromatography coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry (RRLC/Q-TOF-MS).

During the past two decades, RRLC/Q-TOF-MS had been extensively developed for the identification and quantification of components in the complex matrix (Ni et al, 2010; Zhou et al, 2009; Jeanville et al, 2000). RRLC/Q-TOF-MS combines the highly efficient separation capability of RRLC and the high accuracy of Q-TOF-MS. The objective in the present paper is to develop a rapid and simple method using RRLC/Q-TOF-MS for the determination of protopine in brain tissues of rats. The developed method is further validated by assay specificity, linearity, accuracy, precision, extraction recovery, matrix effects, and stability. The results demonstrated that the proposed methodology was suitable for the study on brain tissues of rats. Moreover, this method has been successfully applied for the determination of protopine in brain tissues of rats after ig administration of CDR extract.

2. Materials and methods

2.1 Chemicals and materials

The raw materials of *Corydalis Decumbentis Rhizoma* were collected from Yujiang, Jiangxi province. Protopine and sinomenine (Figure 1) were purchased from the National Institute for Food and Drug Control (Beijing, China), and the purity of the reference substance was determined to be higher than 98% by normalization of the peak area by RRLC/Q-TOF-MS. Acetonitrile of HPLC grade was supplied by Merck (Darmstadt, Germany).

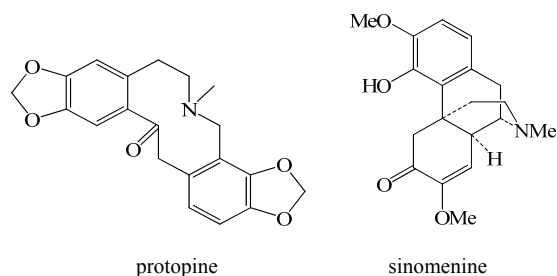


Figure 1 Chemical structures of protopine and sinomenine

Formic acid (purity of 96%) was purchased from Tedia (USA). Deionized water was purified by Milli-Q Plus Water Purification System (Millipore, USA) and filtered with 0.22 μm membranes. All other reagents were of analytical grade and purchased from Shanghai Chemical Reagent Company (Shanghai, China).

2.2 Preparation of CDR extract

The dried and powdered CDR (300 g) was refluxed with 2.0 L of 95% ethanol solution for 2 h and filtered using analytical filter paper. Then the residue was refluxed again with 2.0 L of 95% ethanol under the same conditions. The two extracts were merged and evaporated by rotary evaporation under vacuum at 45 $^{\circ}\text{C}$. The content of protopine is 2.3% in the dried residue.

2.3 LC-MS instrument and conditions

LC analysis was performed on Agilent 1200 Series LC System equipped with binary pump, online degasser, auto plate-sampler, and thermostatically controlled column compartment (Agilent Technologies, Germany). The chromatographic separation was carried out at 30 $^{\circ}\text{C}$ on an Agilent Zorbax Extend- C_{18} column (100 mm $\times$ 3.0 mm, 1.8 μm). The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile (B) using a gradient elution of 10%–25% B at 0–10 min, 25%–100% B at 10–25 min, and 100% B at 25–30 min. The flow rate was kept at 0.4 mL/min.

MS was performed using an Agilent 6538 Q-TOF-MS equipped with an electrospray ionization (ESI) interface (Agilent Technologies, Germany). The experiments are operated in the positive MS scan mode, using the following operating parameters: drying gas (N_2) flow rate of 9.0 L/min, drying gas temperature of 350 $^{\circ}\text{C}$, nebulizer of 30 psig, capillary of 4000 V, skimmer of 65 V, OCT 1 RF Vpp of 750 V, and fragmentor voltage of 170 V. Data acquisition was accomplished with Agilent Mass Hunter Acquisition Software (version B.03.01). Mass spectra were recorded across the range of m/z 100–1700 with accurate mass measurement of all mass peaks.

2.4 Sample preparation

A liquid-liquid extraction technique was applied to isolating protopine and the internal standard (IS, the final concentration was 1.27 $\mu\text{g/mL}$) sinomenine from the homogenate samples of brain tissue. Brain tissue homogenate (500 μL) was extracted with dichloromethane (3.0 mL $\times$ 2) after addition of 10 μL IS solution, and then the dichloromethane fraction was evaporated to dryness under a nitrogen stream in water bath (45 $^{\circ}\text{C}$) away from light. The residue was resolved in 200 μL methanol, and then 1 μL of the supernatant was injected into the LC-MS system. The peak area ratio of protopine to IS was used for the quantitative analysis.

2.5 Preparation of calibration standards and quality control samples

Stock standard solutions of protopine and IS were prepared in methanol at the concentration of 11.96 $\mu\text{g/mL}$ and 2.53 mg/mL, respectively. Working solutions of analytes between 119.6 and 3588 ng/mL were prepared by diluting the stock solution with methanol. The working solution of IS with a concentration of 25.3 $\mu\text{g/mL}$ was obtained in the same way.

Calibration samples, at the concentration of 12.0, 59.8, 119.6, 299, 598, and 897 ng/mL, were prepared by freshly spiking 50 μ L working solutions into 500 μ L blank brain tissue homogenate, and processed as described in the sample preparation. Quality control (QC) samples (598, 119.6, and 12.0 ng/mL) were obtained in the same way as the preparation of calibration standard samples. All solutions were stored at 4 °C.

2.6 Method validation

2.6.1 Assay specificity

The specificity of the method was evaluated by comparing blank brain homogenate samples collected from six different rats with the corresponding drug-brain homogenate samples. Each blank sample was tested for endogenous interference using the proposed extraction procedure and LC-MS conditions.

2.6.2 Linearity and lower limit of quantification (LLOQ)

The calibration curve of protopine was prepared and assayed by the above mentioned method. The calibration curve was constructed by the plots of the peak-area ratios of protopine / IS (y) against the concentration of standards (x). The concentration of protopine in unknown samples was determined by interpolation from the calibration curve. The LLOQ was defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%.

2.6.3 Accuracy and precision

The accuracy and precision of this analytical method were evaluated using QC samples ($n = 5$). Accuracy and precision were respectively expressed as the relative error (RE) and relative standard deviation (RSD). Method precision was checked by intra- and inter-day variability. The intra-day variability was determined by measuring three QC samples for six consecutive times in the same day. The inter-day variability was determined for three consecutive days. The concentration of each sample was calculated using a calibration curve constructed on the same testing day.

2.6.4 Extraction recovery and matrix effects

The extraction recovery of protopine was determined by comparing the peak area of protopine in the extracted QC samples with those obtained from the extracted blank brain tissue samples post-spiked with corresponding neat solutions ($n = 5$). The recovery of IS was determined in a similar way.

The matrix effect (ME) was examined by comparing the mean peak areas of protopine and IS between two different sets of samples. In set 1, reference standards were dissolved in mobile phase and five replicates were analyzed. In set 2, the blank brain tissue homogenate samples were extracted and then spiked with the same concentration of protopine and IS. Samples of both sets were prepared at three QC levels for the analytes. The blank brain tissue homogenate samples used in this study were from five different batches of blank brain tissues. The ME was defined as following: $ME / \% = \text{set 2} /$

set 1 $\times 100\%$. It would indicate the possibility of ionization suppression or enhancement for protopine and IS.

2.6.5 Stability

The stability of protopine in brain tissues was estimated by QC samples using five replicates. The stability validation of protopine was investigated at different conditions. Freeze-thaw stability of protopine was determined in freeze-thaw cycles during 3 d. In each freeze-thaw cycle, the spiked brain tissue samples were frozen for 24 h at $-20\text{ }^{\circ}\text{C}$ and thawed at room temperature ($25\text{ }^{\circ}\text{C}$). The long-term stability was estimated after storage of spiked QC samples at $-20\text{ }^{\circ}\text{C}$ for 2 weeks. The short-term stability was determined by exposing the samples at room temperature for 4 and 12 h.

2.7 Brain tissue distribution of protopine in rats

Animal experiments were carried out according to *The National Institutes of Health Guide for Care and Use of Laboratory Animals*, and were approved by the Bioethics Committee of Kanion Pharmaceutical Co., Ltd.

A brain tissue distribution study was performed to show the applicability of newly developed and validated method. The study was performed in male Sprague-Dawley rats (180–200) g. They were kept in an environmentally controlled breeding room at $(22 \pm 2)\text{ }^{\circ}\text{C}$ with the relative humidity of $(50 \pm 10)\%$, and provided with standard laboratory food and water. They were allowed to adapt to the housing environment for at least 1 week before the study. The experimental animals were deprived of food for 12 h once more.

The CDR extract was suspended in 0.2% carboxymethyl cellulose sodium (CMC-Na) aqueous solution and was ig administered to the rats at a dose of 16 mg/kg for protopine. Five rats for each time point were sacrificed by cervical dislocation immediately at 0.5, 1, 2, 4, 6, 8, 10, 12, 14, and 16 h following ig administration and the whole brain tissue samples were dissected. Brain tissues were washed with physiological saline to remove blood. After drying on filter paper, the brain tissues were weighed and homogenized in saline (1:2). Five rats without any treatment were sacrificed and brain tissues were collected in the same way for the method development and validation. Brain tissue homogenate samples were stored at $-20\text{ }^{\circ}\text{C}$ until analysis.

2.8 Data analysis

The data was analyzed in Microsoft Excel. The results were expressed as $\bar{x} \pm s$.

3. Results and discussion

3.1 Optimization of RRLC/Q-TOF-MS for quantitative analysis

To optimize ESI conditions for the detection of analytes, both the positive and negative ion modes were investigated. The results showed that the response of positive ion mode

was much more sensitive than that of negative ion mode for protopine and IS. Under the selected source condition, the m/z 354.13 for protopine and m/z 330.17 for IS were selected for further LC-MS analysis. The positive ion mass scan spectra of the protopine and IS are shown in Figure 2.

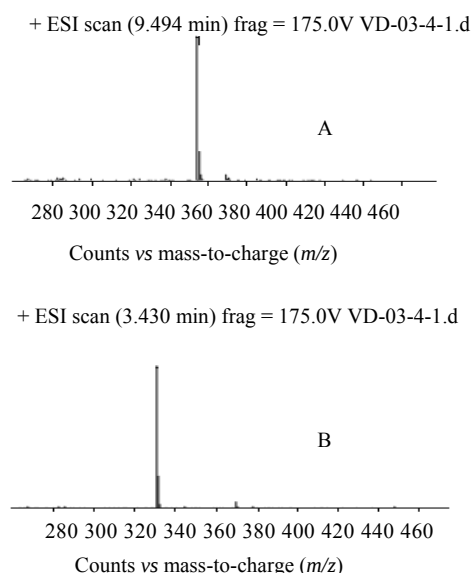


Figure 2 Mass scan spectra of protopine (A) and IS (B) in positive ion mode of RRLC/Q-TOF-MS

3.2 Sample preparation

The aim of sample preparation is to remove interferences from biological samples with a suitable recovery and simple procedure. During the development of biological sample preparation techniques, liquid-liquid extraction, protein precipitation, and solid-phase extraction are the most widely employed methods. The three methods were investigated and compared. It was shown that liquid-liquid extraction by dichloromethane provided higher recovery. The advantage of liquid-liquid extraction is that it minimizes the chances of errors, saves significant time, and simplifies the sample preparation procedure.

The appropriate IS is needed for the accurate quantitative analysis of protopine in biological samples using LC-MS. Several compounds were investigated to find the suitable IS. Sinomenine was finally selected because of its similar chromatographic, MS, and extraction behaviors with protopine.

3.3 Method validation

3.3.1 Specificity

Under the optimal LC-MS conditions, the representative extract ion chromatograms (EICs) of blank rat brain tissue, blank sample spiked with protopine and IS, and sample obtained at 6 h after ig administration are shown in Figure 3. The typical retention time for protopine and IS was 9.5 and

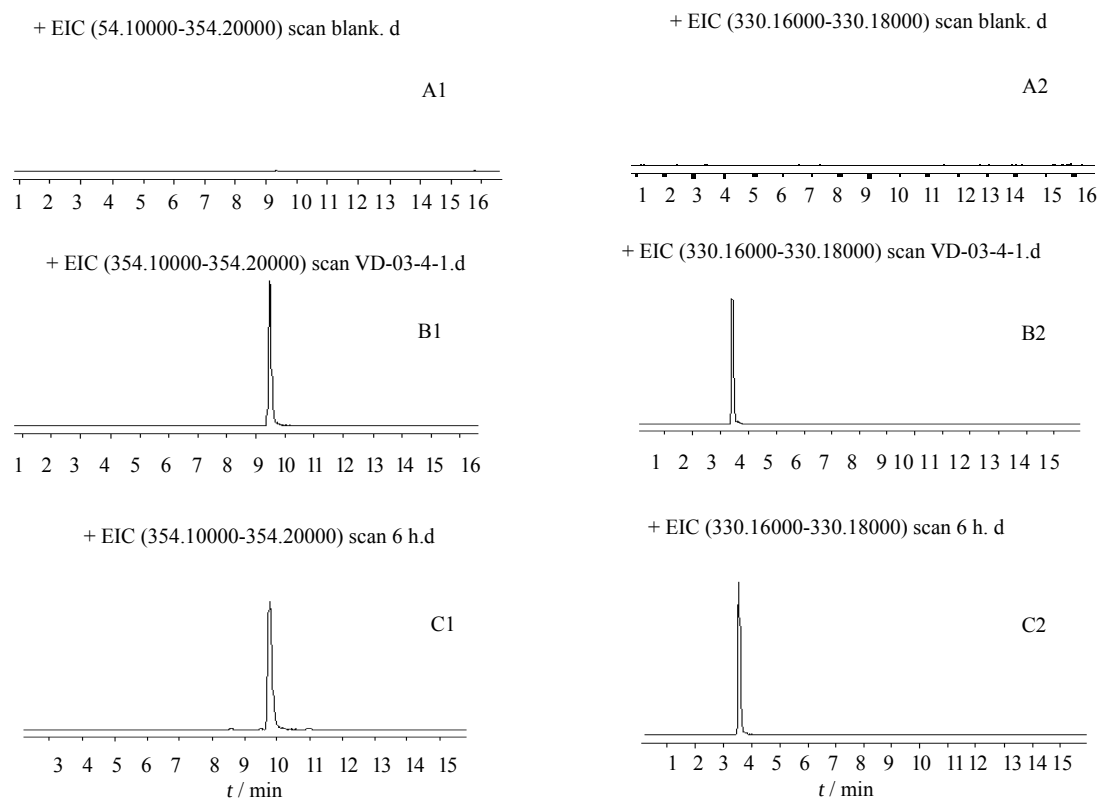


Figure 3 EICs of protopine (1) and IS (2) in blank rat brain tissues (A), in rat brain samples spiked with protopine and IS (B), and in rat brain samples of 6 h after ig administration spiked with IS (C)

3.43 min, respectively. No obvious endogenous substances was observed at the retention time of protopine and IS in the matrix.

3.3.2 Linearity and LLOQ

The calibration curve for protopine was linear in the concentration range of 12–897 ng/mL in brain tissue homogenate of rats, and the regression equation was $y = 0.0015x + 0.0096$. The correlation coefficient (r) for each calibration curve was over 0.998.

The LLOQ was 12 ng/mL for protopine. This limit was sufficient to determine the concentration-time profile of protopine in rat brain following ig administration of CDR.

3.3.3 Accuracy and precision

The results of accuracy and precision are presented in Table 1, and the RSD values obtained from intra- and inter-day precision studies are less than 10%. The deviation of assay accuracies ranged from –9.0% to 6.8%. The results indicated that the method was reliable and reproducible for the quantitative analysis of protopine in brain tissue homogenate samples of rats.

Table 1 Intra- and inter-day assay for accuracy and precision of protopine in brain tissue homogenate of rats ($n = 5$)

Precision	QC concentration / (ng·mL ⁻¹)	C_{det} / (ng·mL ⁻¹)	RSD / %	RE / %
intra-day	598.0	633.0 ± 63.0	10	5.9
	119.6	117.2 ± 9.0	7.7	-2.0
	12.0	11.0 ± 0.03	0.30	-9.0
inter-day	598.0	639.0 ± 63.0	9.9	6.8
	119.6	116.7 ± 9.9	8.5	-2.4
	12.0	11.0 ± 1.0	10.9	-8.7

3.3.4 Recovery and matrix effect

As shown in Table 2, the recoveries of protopine were (96.4 ± 0.03)%, (99.6 ± 0.07)%, and (98.5 ± 0.08)% at three QC concentration levels, respectively. The recovery of IS (1.27 µg/mL) was (98.6 ± 0.02)%. It indicated that the extraction efficiency of protopine using liquid-liquid extraction was sufficient and was not concentration-dependent.

Table 2 Extraction recoveries for analysis of protopine in brain tissues of rats ($n = 5$)

Sample	QC concentration / (ng·mL ⁻¹)	Recoveries / %
protopine	598.0	96.4 ± 0.0
	119.6	99.6 ± 0.0
	12.0	98.5 ± 0.0
IS	1270.0	98.6 ± 0.0

The average matrix effect values were (99.7 ± 0.65)%, (98.1 ± 0.43)%, and (100.4 ± 0.80)% for protopine at the three QC concentration levels. The average matrix effect value of IS was (91.7 ± 0.72)%. It concluded that the extracts had little detectable endogenous substances that could influence the ionization of protopine and IS.

3.3.5 Stability

The stability of protopine in brain tissue homogenate samples of rats under different storage conditions is summarized in Table 3. The results demonstrated that protopine was stable at –20 °C for 14 d. Treated samples were found to be stable at 25 °C for 12 h. No degradation was observed after three freeze-thaw cycles. These studies suggested that the brain tissue homogenate samples containing protopine could be handled under normal laboratory conditions without significant degradation.

Table 3 Stability of protopine in brain tissues of rats under different storage conditions ($n = 5$)

QC concentration / (ng·mL ⁻¹)	Short-term stability / (25 °C, 4 h)		Short-term stability / (25 °C, 12 h)		Three freeze-thaw cycles		Long-term stability / (–20 °C, 14 d)	
	RSD/%	RE/%	RSD/%	RE/%	RSD/%	RE/%	RSD/%	RE/%
598	3.6	7.0	4.5	11.5	4.3	9.1	4.6	6.5
119.6	3.8	9.4	4.7	8.6	7.7	7.2	4.9	0.8
12	5	13.5	3.4	12.8	3.4	9.6	9.2	8.7

3.4 Brain tissue distribution study of protopine in rats

The validated method was successfully applied to the determination of protopine in brain tissues of rats. The mean rat brain concentration-time curve of protopine is shown in Figure 4. Following ig administration of CDR extract, significant amount of protopine could reach rat brain rapidly within 0.5 h. It is suggested that protopine possesses appreciable blood-brain barrier penetrability, and it supports its beneficial anti-demented effect. So protopine may be more effective in the treatment of dementia. The information described above might be helpful for further studies on the pharmacokinetics of CDR, and beneficial for application of the CMM in clinical therapy.

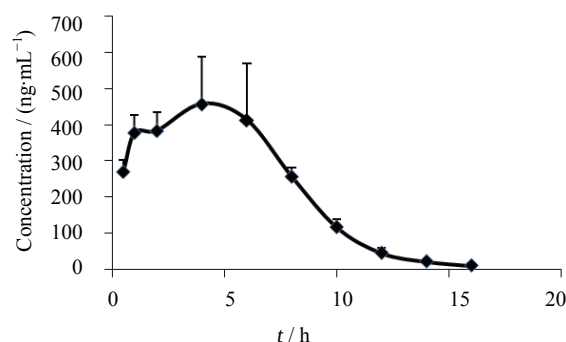


Figure 4 Brain tissue concentration-time curve of protopine in rats after ig administration of CDR (16 mg/kg) ($n = 5$)

4. Conclusion

In this paper a sensitive and reliable RRLC-Q-TOF-MS method has been developed for the determination of protopine in rat brain. This method has significant advantages in terms of simple precipitation procedure and short chromatographic running time (9.5 min). The method could give the consistent and reproducible recoveries for protopine and IS from rat brain with minimum interference. The method is sensitive with a LLOQ of 12 ng/mL. The established RRLC-Q-TOF-MS method for studying the brain tissue distribution of protopine in rats including chromatographic conditions as well as sample preparation procedures is very useful for future study in protopine related drug development. It also could facilitate, with minor modification, the development and validation of RRLC-Q-TOF-MS analytical assays to analyze protopine in other biological matrixes such as urine, plasma, and other tissues.

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