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Application of hollow fiber liquid phase microextraction coupled with high-performance liquid chromatography for the study of the osthole pharmacokinetics in cerebral ischemia hypoperfusion rat plasma

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ABSTRACT

A simple and solvent-minimized sample preparation technique based on two-phase hollow fiber liquid phase microextraction has been developed and used to quantify the osthole in cerebral ischemia reperfusion rat plasma following oral administration. The analysis was performed by reversed phase high performance liquid chromatography with fluorescence detection. Extraction conditions such as solvent identity, agitation rate, salt concentration, extraction time, and length of the hollow fiber were optimized. Under the optimized conditions, the linear range of osthole in rat plasma was 5–500 ng mL^{-1} (r^2 = 0.9997). The limit of detection (LOD) was 2 ng mL^{-1} (S/N = 3) with limit of quantification (LOQ) being 5 ng mL^{-1}. The validated method has been successfully applied for pharmacokinetic studies of osthole from cerebral ischemia reperfusion rat plasma after oral administration.

1. Introduction

Osthole (Fig. 1A), a component isolated from medicinal plants, such as *Cnidium monnieri* (Chinese name: She Chuang Zi), is a natural coumarin derivative. It exerts a broad spectrum of pharmacological activities including anti-osteoporotic [1,2], anti-proliferative [3], anti-allergic [4,5], anti-seizure [6] and anti-diabetic [7] effects. Recently, Ji et al. studied the neuroprotective effects and mechanisms of osthole on chronic cerebral hypoperfusion in rats [8]. Chronic cerebral hypoperfusion has been well characterized as a common pathological status contributin to neurodegenerative diseases such as vascular dementia [9–11]. The main clinical outcomes of this disease are the cognitive deficits and permanent neural impairment [12]. Therefore, the potency of osthole makes it a promising candidate for development as a novel anti-cerebral ischemia drug. In order to provide valuable information of quantification and pharmacokinetics studies on osthole in cerebral vascular disease, a simple, rapid and reliable analysis method should been developed. Although, several methods have been developed for the analysis of osthole in plasma by HPLC [13–16]. To our knowledge, this is the first report of the combined use of HP-LPME with HPLC for the trace analysis of osthole in plasma.

For the analysis of osthole in biological samples, sample preparation is a critical step in the analytical procedure. Although, Various sample preparation methods have been established for extraction and preconcentration of osthole from the matrix, such as liquid–liquid extraction (LLE) [13,14], solid-phase extraction (SPE) [15] and cloud point extraction (CPE) [16], but the disadvantages such as intensive labor, time consuming, unsatisfactory enrichment factor and large quantity of toxic solvent, limit their application. Recently, hollow fiber protected liquid-phase microextraction (HF-LPME) was developed as a promising method in samples preparation owing to its simplicity, efficiency, low cost, negligible volume of solvents used and excellent sample cleanup ability. The basic principle of this technique has been described clearly in previous publications [17,18]. In brief, there are two modes used: two-phase HF-LPME and three-phase HF-LPME. A piece of porous polypropylene hollow fiber is firstly placed in the aqueous sample and the analytes are extracted by passive diffusion from the sample into the hydrophobic organic solvent supported by the fiber (acceptor phase, two-phase HF-LPME). Alternatively, the analytes were extracted through an organic solvent immobilized in the pores of fiber and further into a new aqueous phase in the lumen of fiber (acceptor phase, three-phase HF-LPME). In general, two-phase HF-LPME is applied for analytes with a high solubility in non-polar organic solvents and three-phase HF-LPME is applied for basic or acidic analytes with ionisable functionalities. HF-LPME has been

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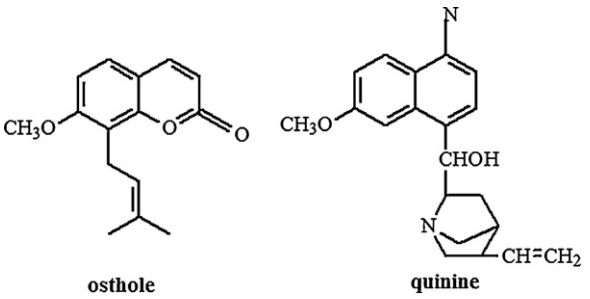

Fig. 1. Chemical structures of osthole (A) and quinine (B) (internal standard).

successfully applied to the extraction of drugs from a variety of biological fluids and to the preconcentration of pollutants from several environmental matrices [19–21].

A successful HF-LPME could not only extract target analyte efficiently but also prevent proteins and the majority of other endogenic compounds in the biological samples from entering into the organic phase and acceptor phase [22]. In this paper, two-phase HF-LPME was applied for the extraction and determination of osthole in rat plasma. Optimum conditions for this method were examined. The feasibility of this methodology is also evaluated by determining the enrichment factor, linearity, detection limit and recovery. Overall, this method proves to be an attractive alternative to other procedures having the advantages of being simple, inexpensive, sensitive, fast and requiring little solvent. As the cost of analysis per sample is low, the hollow fiber can be disposed off after a single extraction, thus avoiding the possibility of carry-over between analyses.

2. Experimental

2.1. Chemicals and reagents

Osthole ($C_{15}H_{16}O_3$, MW = 244.29) and the internal standard (IS) (quinine) ($C_{20}H_{24}N_2O_2$, MW = 324.42) (purity > 98%) were purchased from the National Institute for the Control of Pharmaceutical and Biological products (Beijing, China). The structure of osthole and quinine are shown in Fig. 1. Osthole was dissolved in olive oil and administrated p.o. at a volume of 10 mL kg^{-1}. Dodecanol, benzylalcohol, *n*-decanol, *n*-octanol, toluene and *n*-hexane were obtained from Merck (Darmstadt, Hessen, Germany). Phosphoric acid (Beijing Chemical Factory, AR Beijing, China) was prepared before experiment. Acetonitrile was of HPLC-grade and obtained from Fisher (Pittsburgh, Pennsylvania, USA). All other reagents used in this work were of analytical grade. Ultrapure water (Millipore, Bedford, Ohio, USA) was used throughout the study.

Stock solutions of osthole (10 μg mL^{-1}) and IS (2 μg mL^{-1}) were prepared by dissolving suitable amounts of each pure substance in methanol–water (60:40, v/v) and kept stable for 2 months when stored at 4 °C in the refrigerator (assessed by HPLC). The stock solutions and biological samples were all kept protected from light and 10 μL internal standard solution was added into each plasma sample (the final concentrations of IS were 50 ng mL^{-1}) prior to extraction.

2.2. Animals and model of cerebral ischemia hypoperfusion

All experimental protocols were approved by the Institutional Animal Care and Use Committee of XinJiang Medical University, and performed in accordance with the National Institutes of Health (NIH, USA) guidelines for the use of experimental animals. Adult Male Wister rats (230 ± 20 g), provided by the Laboratory Animal Center of XinJiang Medical University, and were used in all experiments. Animals were housed in a temperature-and humidity-controlled room that was maintained on 12 h light/dark cycles for at least 1 week before surgery. Standard animal chow and water were freely available. All efforts were made to minimize animal suffering in this study.

Six rats were anesthetized with choral hydrate (350 mg kg^{-1}, i.p.), the bilateral common carotid arteries of the rats were exposed and carefully separated from carotid sheath, cervical sympathetic and vagal nerves through a ventral cervical incision. The bilateral common carotid arteries were ligated with 4-0 type surgical silk in ischemia rats. The operation was performed on a heating pad to maintain body temperature at 37.5 ± 0.5 °C. The animal was kept on the pad until recovery from anesthesia.

2.3. HPLC system

All analyses were performed on an Dionex HPLC system (Sunnyvale, CA, USA) which consisted of a P680 quaternary pump, TCC-100 thermostatted column compartment, RF-2000 Fluorescence detector and Rheodyne 7225i injector. The chromatography data were recorded and processed with Dionex Chromeleon 6.8 Chromatography Software. Chromatographic separations were achieved on an Dionex Acclaim-C_{18} (75 mm × 4.6 mm i.d., 3.5 μm) column connected with an Agilent Zorbax Extend-C_{18} guard column (Wilmington, NC, USA) (12.5 mm × 2.1 mm i.d., 3.5 μm) kept at 25 °C. Fluorescence detection was performed with excitation and emission wave-lengths set at 325 and 405 nm, respectively.

The mobile phase was a gradient elution of A (0.1% phosphoric acid) and B (acetonitrile) was used. The linear gradient was as follows: 5–10% B over 0–3 min, 10–30% B over 3–6 min, 30–60% B over 6–9 min and then returned to 5% B at 9 min immediately. The flow rate was set at 1.0 mL min^{-1}. The injections were carried out through a 10 μL loop. Retention data were recorded using the described above chromatographic conditions. Column void volume was determined to be 1.15 min by the injection of acetone. Retention behavior of the analytes was estimated by retention factor (k) and calculated according to the equation, $k = (t_R - t_0)/t_0$, where t_R is the retention time of the analyte and t_0 is the elution time of the acetone (as a void marker) [23].

2.4. Blood sample preparation

Rats were anesthesized by pentobarbital sodium and blood (1.0 mL) was collected from abdominal artery in clean heparinized glass tubes. The blood samples were separated by immediate centrifuging at 3500 rpm for 5 min. The plasma obtained was stored frozen at −20 °C until analysis.

2.4.1. HF-LPME procedure

The Accurel Q3/2 polypropylene hollow fiber used for LPME was purchased from Membrana (Wuppertal, Germany). The inner diameter was 600 μm, the pore size was 0.2 μm and the thickness of the wall was 200 μm. Before use, the hollow fiber was ultrasonically cleaned in acetone for 4 min in order to remove any contaminants. After drying, the hollow fiber was cut manually into segments 4 cm long. In order to decrease the memory effect, each segment was used once.

The needle tip was inserted into the hollow fiber, and the assembly was immersed in the organic solvent for around 10 s in order for the solvent to impregnate the pores of the fiber wall. Since the hollow fiber is hydrophobic, the fiber channel could be filled with organic solvent. 0.2 mL plasma sample and 10 μL of internal standard solution (50 ng mL^{-1}) were placed in a conventional vial with a screw top/silicone septum (Supelco, Bellefonte, PA, USA). The plasma sample was diluted with ultrapure water to a total volume of 5 mL containing 1.5% (w/v) NaCl. The whole fiber is totally

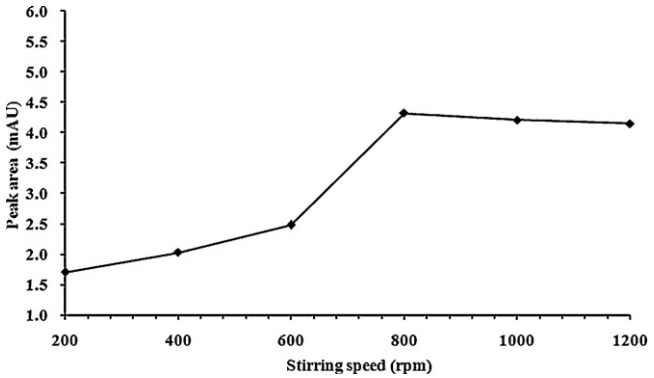

Fig. 2. Effect of stirring speed on extraction recovery of osthole. Conditions: osthole, 50 ng mL^{-1}; organic extraction solvent, *n*-octanol; extraction time, 20 min; concentration of sodium chloride solution, 1.5% (w/v); length of the fiber, 4 cm.

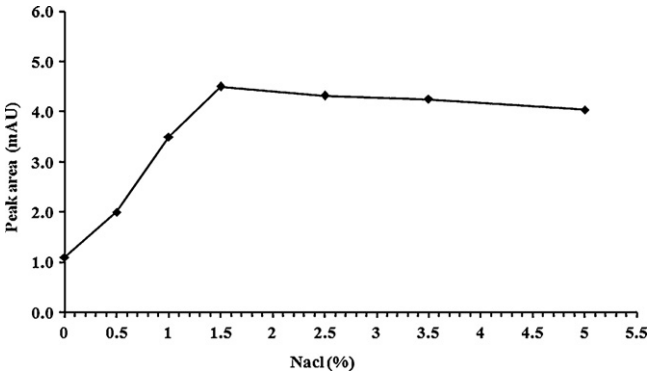

Fig. 3. Effect of the salt on the extraction efficiency. Conditions: osthole, 50 ng mL^{-1}; organic extraction solvent, *n*-octanol; extraction time, 20 min; stirring rate, 800 rpm; length of the fiber, 4 cm.

immersed in the water phase but above the magnetic stirrer bar, so it would not be damaged during the stirring. Finally, the organic solvent (12 μL) (acceptor phase) in the syringe was injected carefully and completely into the hollow fiber. The sample was continuously stirred at room temperature (25 °C) with a magnetic stirrer to facilitate the mass transfer process and to decrease the time required for the equilibrium to be established. After 20 min extraction, the analyte-enriched solvent (10 μL) was withdrawn into the syringe, the fiber segment was removed and the organic phase was then injected into the HPLC for further analysis.

Enrichment factor (*EF*) was defined as the ratio between the slope of standard curve after and before extraction. The extraction recovery (*R*) was calculated by the following equations:

$$R = \left(\frac{V_a}{V_s}\right)(EF) \times 100\%$$

where V_a was the volume of the acceptor phase (12 μL), V_s was the volume of the diluted sample (5 mL).

2.4.2. CPE procedure

200 μL of rat plasma sample and 10 μL of internal standard solution (50 ng mL^{-1}) were added to a 1.5 mL capped centrifugal tube. To this were added 1 mL of aqueous solution of Triton X-114 at concentration of 0.8% (w/v) and 100 μL 0.4 M sodium chloride solutions. The contents were mixed well with a Vortex Genie Mixture (CAY-1, Beijing Changan Instrumental Factory, PR China) for 5 min, and then incubated in the thermostatic bath at 45 °C for 20 min. The phase separation was then accelerated by centrifugation at 3500 rpm for 5 min. After removing of the water phase, a surfactant-rich phase stuck to the bottom of the tube was obtained. Coextractants such as hydrophobic proteins and most of the surfactant were removed from the surfactant-rich phase by precipitation with 200 μL of acetonitrile–water (30:70, v/v), vortex-mixed and centrifuged at 16,000 rpm for 10 min. 10 μL volume of this sample solution was injected onto HPLC for analysis [16].

2.5. Method validation

2.5.1. Calibration curve

By spiking the appropriate stock solution containing the IS at a constant concentration to 0.2 mL of blank plasma, six effective concentrations (5, 10, 25, 50, 100, 250, 500 ng mL^{-1}) for osthole were obtained separately. The quality control (QC) samples were prepared in blank plasma at the concentrations of 5, 50, 500 ng mL^{-1} containing the IS at a constant concentration, respectively. The spiked plasma samples (standards and quality controls) were then treated following the previously described procedure and injected into the HPLC. The procedure was carried out in triplicate for each concentration. The obtained analyte/IS peak area ratios were plotted against the corresponding concentrations of osthole and the calibration curves were set up by the least-squares method. The values of limit of quantification (LOQ) and limit of detection (LOD) were calculated, according to Chinese Pharmacopoeia [24] guidelines. The analyte concentrations gave rise to peaks whose heights were 10 and 3 times the baseline noise, respectively.

2.5.2. Extraction recovery (absolute recovery)

By assaying the samples at three QC levels, absolute recoveries of osthole were determined. The analyte/IS peak area ratios were compared to those obtained from the direct injection of the compounds dissolved in the organic solvent (acceptor phase) of the processed blank plasma at the same theoretical concentrations. The extraction recovery values were calculated as follows:

$$\frac{(\text{analyte/IS peak area ratio})_{\text{spiked blank}}}{(\text{analyte/IS peak area ratio})_{\text{corresponding standard}}} \times 100\%$$

2.5.3. Precision and accuracy

The precision, including intra-day and inter-day precision expressed as % R.S.D. values, was assessed by assaying the samples at three QC levels. The intra-day variance was determined by assaying the spiked samples five times during one day with the inter-day variance assayed five consecutive days. The accuracy was evaluated by mean recovery and expressed as (mean measured concentration)/(spiked concentration) × 100% and % R.S.D. values.

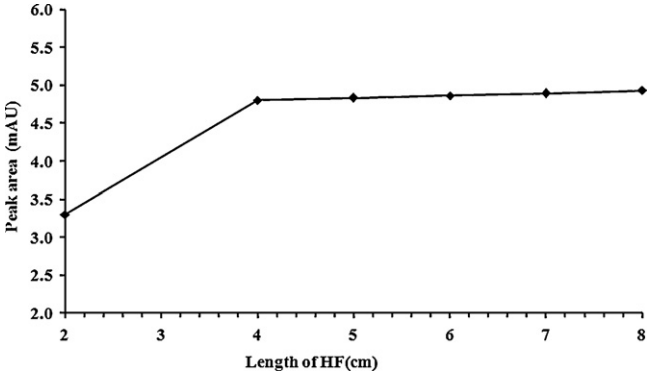

Fig. 4. Effect of hollow fiber length on LPME efficiency. Conditions: osthole, 50 ng mL^{-1}; organic extraction solvent, *n*-octanol; extraction time, 20 min; stirring rate, 800 rpm; concentration of sodium chloride solution, 1.5% (w/v).

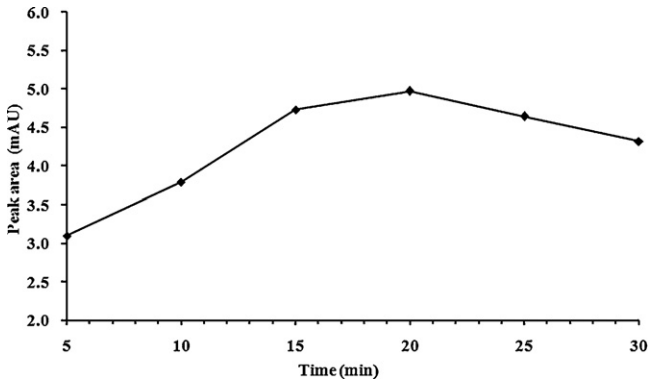

Fig. 5. Effect of extraction time on extraction recovery of osthole. Conditions: osthole, 50 ng mL^{-1}; organic extraction solvent, *n*-octanol; length of the fiber, 4 cm; stirring rate, 800 rpm; concentration of sodium chloride solution, 1.5% (w/v).

2.5.4. Selectivity

Blank plasma and drug plasma samples from rats were injected into the HPLC. The resulting chromatograms were checked for possible interference from endogenous substances and metabolites of osthole. The acceptance criterion was no interfering peak in the place of an analyte peak.

2.5.5. Stability

To evaluate sample stability after freeze–thaw cycles and at room temperature, five replicates of QC samples at each of 20, 1000 and 2000 ng mL^{-1} concentrations were subjected to three freeze–thaw (−20 to 25 °C) cycles or were stored at room temperature (approximately 22–25 °C) for 4 h before sample processing, respectively. Long-term stability was studied by assaying samples that had been stored at −20 °C for a certain period of time (15 day). Stability was assessed by comparing the mean concentration of the stored QC samples with the mean concentration of those prepared freshly. Ampelopsin was considered stable under storage conditions if the assay percent recovery was found to be 85–115% of the nominal initial concentration [25].

2.6. Application to pharmacokinetic study

The cerebral ischemia hypoperfusion was operated by occupation of bilateral carotids for 30 min, prior to osthole administration. A weight matched group of six rats was left intact served as the normal control. Osthole was orally administrated to rats at a dose of 20 mg kg^{-1} and blood samples were collected at times of 0.083, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h after dosing. Then the samples were centrifuged and the separated plasma samples were processed according to the above-mentioned method. Data from these samples were used to construct pharmacokinetic profiles by plotting drug concentration versus time. All data were subsequently processed by DAS 2.0 statistical software (Pharmacology Institute of China). Data were expressed as means ± SD. The student's *t*-test assesses the statistical significance which was set at $P < 0.05$.

Table 1
Inter-day precision in the slope, intercept and correlation coeffcient (*r*) of standard curves (*r* = 0.9996–0.9999).

Day	Slope	Intercept	*r*
1	0.00626	0.4982	0.9996
2	0.00612	0.4901	0.9999
3	0.00641	0.5044	0.9996
4	0.00634	0.5003	0.9998
5	0.00642	0.4972	0.9997
Mean ± SD	0.00631 ± 0.000124	0.4982 ± 0.00524	0.9997 ± 0.00013
C.V. (%)	1.96	1.05	0.01

C.V., co-efficient of variation.

Table 2
Accuracy and precision for the assay of osthole in rat plasma (*n* = 5).

Theoretical concentration (ng mL)	Assayed concentration (ng mL) (mean ± SD)	Accuracy (%)	Precision (R.S.D.%)
Intra-day			
5	4.7 ± 0.32	94.0	6.1
50	48.2 ± 6.85	96.4	4.5
500	486.5 ± ll.51	97.3	2.8
Inter-day			
5	4.8 ± 0.51	96.0	5.6
50	48.4 ± 7.02	96.8	4.6
500	487.9 ± l2.45	97.6	3.9

R.S.D. = relative standard deviation.

3. Results and discussion

3.1. Optimization of the HF-LPME procedure

To develop an HF-LPME method for the determination of osthole in plasma samples, several parameters controlling optimum performance, such as selection of extraction solvent, effect of agitation rate, effect of salt addition, effect of length of the fiber, and effect of extraction time were assessed.

3.1.1. Selection of the organic extraction solvent

The type of organic solvent immobilized in the pores of the hollow fiber is an essential consideration for effcient analyte preconcentration. As in LLE, the principle "like dissolves like" is applied in LPME. The solvent should be of low volatility to prevent evaporation, low viscosity to ensure rapid mass transfer, low polarity to ensure compatibility with the hollow fiber, and to prevent leakage into the sample. In addition, the solvent should provide high distribution constants for the target analytes [26]. Based on the above four considerations, six types of organic solvent (dodecanol, benzylalcohol, *n*-decanol, *n*-octanol, toluene and *n*-hexane) were investigated for use in HF-LPME. 0.2 mL of plasma samples was spiked with osthole at 50 ng mL^{-1}, and the extraction time and agitation rate were 20 min and 800 rpm, respectively. The highest extraction recovery was obtained with *n*-octanol. No enrichment effect of the analyte was observed, especially with toluene and *n*-hexane. Therefore, *n*-octanol was selected as the immobilization solvent for further optimization.

3.1.2. Agitation rate

Sample agitation has a great role to enhance extraction recovery and to reduce extraction time [27]. Agitation permits the continuous exposure of the extraction surface to fresh aqueous sample. Different stirring rates were tested to determine the optimum stirring speed for the extraction. The experiments were carried out at stirring speeds ranging from 200 to 1200 rpm. Fig. 2 shows that extraction recovery reaches the highest value at 800 rpm. Higher spinning rate exceeding 1200 rpm were not evaluated due to excessive air bubbles on the surface of the hollow fiber, which could lead to poorer precision and possible experimental failure. Further experiments were performed with a stirring rate of 800 rpm.

3.1.3. Salt effect

In two-phase LPME, the effect of salt addition to the donor solution prior to extraction has been widely investigated. Depending on the target analytes, an increase in the ionic strength of aqueous solution may have various effects on extraction: it may enhance, not

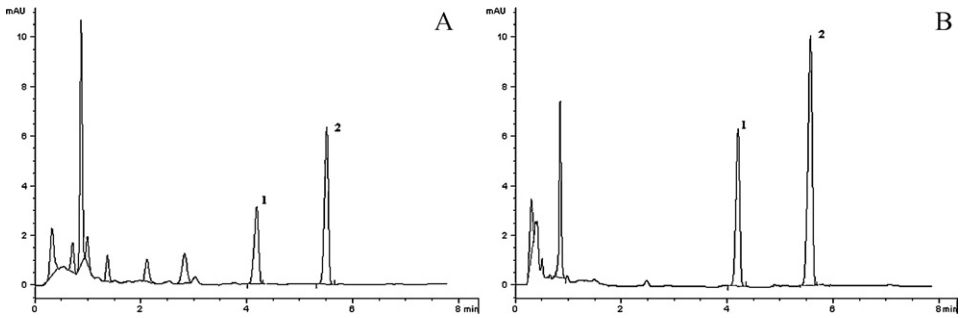

Fig. 6. Typical chromatograms of determination of osthole (50 ng mL^{-1}) and IS in plasma samples; (A) plasma sample 1 h after oral administration with cloud-point extraction; (B) plasma sample 1 h after oral administration with hollow fiber liquid phase microextraction. Peak identification: 1, osthole; 2, IS.

influence or limit extraction [28]. Experiments were performed to investigate the salting out effect on hollow fiber LPME by using 5 mL water samples containing 0, 0.5, 1.5, 2.5, 3.5, and 5% (w/v) NaCl. The extraction recovery for the analyte increased with an increase of salt concentration from 0 to 1.5% (w/v), resulting probably from the salting-out effect (Fig. 3). However, further addition of NaCl did not result in further increase in extraction recovery. It is possible that the high concentration of salt changed the physical properties of the Nernst diffusion film and reduced the rate of diffusion of the analyte into the organic phase [29]. Therefore, 1.5% NaCl (w/v) was used in subsequent experiments.

3.1.4. Effect of length of the fiber

Fiber length is an important factor from the viewpoint of analyte recoveries and sample preparation time. The selection of longer fiber can shorten sample preparation time due to higher surface area, filled the more acceptor into the fiber, and increased the extraction recovery. The effect of hollow fiber length was studied by exposing the hollow fiber of different lengths impregnated with *n*-octanol to the sample solution for 20 min. The sample stirring rate was maintained at 800 rpm. As shown in Fig. 4, The length of the hollow fiber was varied from 2 to 8 cm, and the extraction recovery increased significantly up to 4 cm, whereas for longer fibers, the recovery was unaffected by the fiber length. Based on these results, a hollow fiber of 4 cm length was used for the optimization of the parameters.

3.1.5. Influence of extraction time

Once organic solvent, stirring speed, salt effect and length of the fiber in LPME were fixed, LPME extraction efficiency is determined by extraction time. However, it is usually not practicable to lengthen an extraction for equilibrium to be established. This is because the longer the extraction, the greater the tendency of solvent loss due to dissolution in the sample solution and the formation of air bubbles [30,31]. In this work, the effect of extraction time was evaluated by conducting experiments for 5, 10, 15, 20, 25 and 30 min at a stirring rate of 800 rpm, respectively. The results (Fig. 5) showed that up to 20 min, the extraction recovery of the analyte increased with increased extraction time. However, when the extraction time was longer than 20 min, the extraction recovery decreased. Therefore, an extraction time of 20 min was employed in further experiments.

3.1.6. Optimized extraction procedure

Based on the experiments discussed above, the optimum HF-LPME conditions were as follows: *n*-octanol as organic solvent, 20 min extraction time, 800 rpm stirring rate, 4 cm HF length, and 1.5% (w/v) NaCl content. Under the optimal conditions the enrich factor for the diluted plasma samples were 231 for osthole.

3.2. Calibration and validation

3.2.1. Linearity, limit of detection and limit of quantitation

The calibration curves were constructed by calculating the peak area ratios (Y) of osthole to internal standard against osthole standard concentrations. The calibration curve was $Y = 0.00631 + 0.4982X$ with a correlation coefficient above 0.9997. The mean of five calibration curves made over a period of 5 days, each calibration curve originating from a new set of extractions. Calibration curves were linear in the concentration range investigated with coefficients of correlation (r^2) ≥0.9996. Table 1 shows inter-day precision in the slope, intercept and correlation coefficient of standard curves (r^2 = 0.9996–0.9999) made over a period of 5 days. The coefficient of variation (C.V.) (%) ($n = 5$) of the slope

Table 3
Summary of stability of oslhole in rat plasma ($n = 5$).

Concentration found (ng mL^{-1}) (mean ± SD)	Concentration added (ng mL^{-1}) (mean ± SD)		
	5	50	500
Freeze and thaw stability			
At the beginning	4.8 ± 0J5	48.2 ± 6.26	489.4 ± 10.04
After three freeze–thaw cycle	4.6 ± 0.61	46.8 ± 7.18	479.8 ± 11.63
Bias (R.E.%)	−4.35	−2.99	−2.02
Short-term room temperature stability			
At the beginning	4.8 ± 0.35	48.2 ± 6.26	489.4 ± 10.04
After 4 h at room temperature	4.7 ± 1.19	47.4 ± 6.73	483.5 ± 10.78
Bias[a] (R.E.%)	−2.13	−1.69	−1.12
Long-term cold storage stability			
At the beginning	4.8 ± 0.35	48.2 ± 6.26	489.4 ± 10.04
After 15 days at −20 °C	4.5 ± 0.87	45.8 ± 7.33	471.7 ± 12.31
Bias[a] (R.E.%)	−6.67	−5.24	−3.75

[a] Bias (R.E.%) = $(C_{actual} - C_{calculated})/C_{actual}$ (%).

Table 4
Comparison of figures of merit of the HF-LPME method with CPE method applied for the analysis of osthole.

Method	Sample preparation	LOD (ng mL^{-1})	LR (ng mL^{-1})	Extraction time (min)	*EF*
HPLC-FD	HF-LPME	2	5–500	20	231
HPLC-UV	CPE	30	100–10,000	30	127

calculated with calibration curve data was 1.96%, showing good repeatability. Further evaluations such as residual plots examination and lack of fit test were carried out to check the model's adequacy. No significant lack-of-fit was observed in any of the calibration curves. The correlation coefficient using linear regression model of calibration curve is acceptable ($r^2 = 0.9997$). The limit of LOQ for osthole in plasma was 2 ng mL^{-1} and the limit of LOD was 5 ng mL^{-1}.

3.2.2. Accuracy and precision

The intra-day and inter-day accuracy and precision values of the assay method are shown in Table 2. All intra-day R.S.D. (%) for osthole were below 6.1%. All inter-day R.S.D. (%) were below 5.6%. The accuracies were determined by comparing the mean calculated concentration with the spiked target concentration of the quality control samples. The intra-day and inter-day accuracies for osthole were found to be within 94.0% and 97.6%.

3.2.3. Extraction recovery (absolute recovery)

To determine the recovery of osthole in rat plasma, a blank rat plasma was spiked with osthole to achieve a final concentration of 5, 50, 500 ng mL^{-1}. The plasma samples were subjected to the HF-LPME procedure and injected into the HPLC. Six samples were analyzed for each concentration. The analysis was performed for three replicates at the concentration levels mentioned above. The mean recoveries of osthole from rat plasma at concentrations of 5, 50, 500 ng mL^{-1} were 93.8%, 94.9% and 96.3%. Using the same method, the recovery of I.S. in rat plasma was obtained which was 94.7%.

3.2.4. Selectivity and stability

Selectivity was evaluated by comparing the chromatograms of blank plasma and drug plasma samples, which were subjected to the HF-LPME procedure and injected into the HPLC. Fig. 7 shows the typical chromatograms of a blank plasma sample, of a spiked plasma sample with osthole and IS, and of a plasma sample from 1 h after an oral administration. It also shows no significant interference from endogenous substances and metabolites of osthole observed in the place of the analytes.

Osthole in rat plasma was shown to be stable for at least 15 days stored at −20 °C. The relative error (R.E.) % of osthole in rat plasma between the initial concentrations and the concentrations of the following three freeze–thaw cycles ranged from −4.35 to −2.02%, which indicated that osthole was stable during the three freeze–thaw cycles. Processed samples were also found to be stable for at least 4 h at room temperature. The above stability data are summarized in Table 3. The result shows that no significant deterioration of the analytes was observed under any of these conditions.

3.3. Comparison with cloud-point extraction

Table 4 shows compared figures of merit generated by the HF-LPME method and CPE method for the extraction of osthole from plasma. The advantages of HF-LPME technique were higher EF and shorter extraction time under identical experimental conditions. The linear range (LR) produced by the HF-LPME method shows a wider value and lower LOD in comparison with CPE.

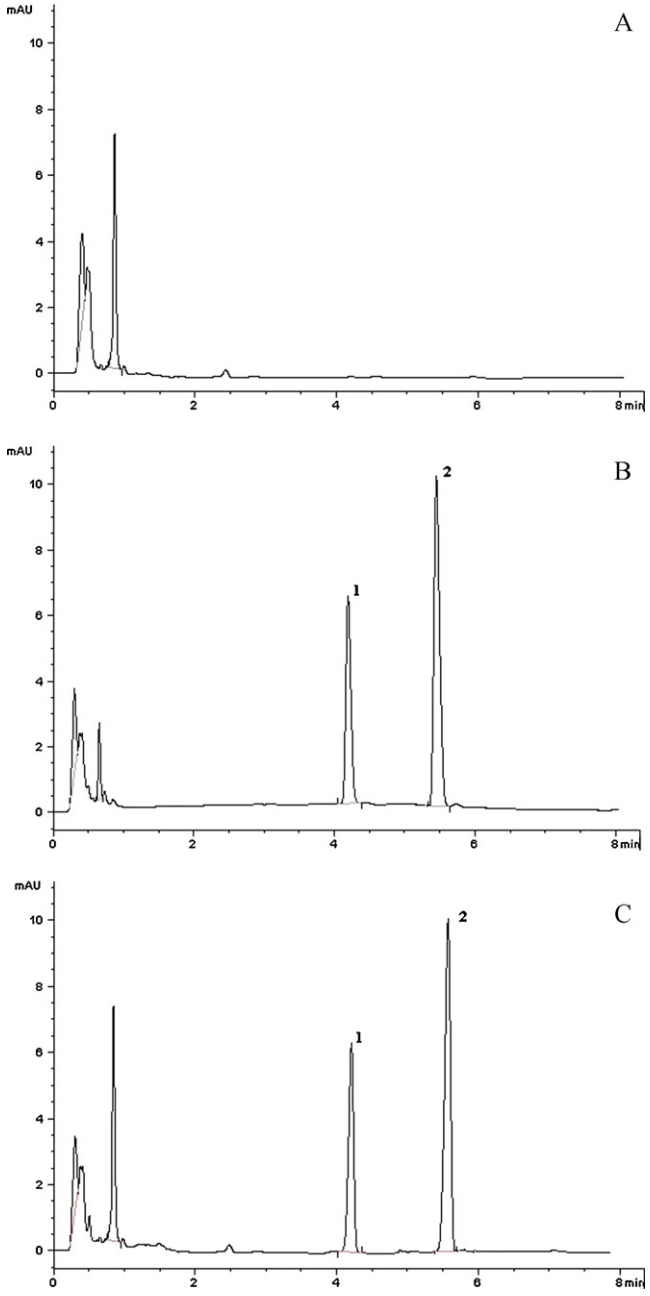

Fig. 7. Typical HPLC chromatograms of a hollow fiber liquid phase microextraction of plasma samples: (A) a blank plasma sample; (B) a blank plasma sample spiked with osthole (50 ng mL^{-1}) and IS; (C) plasma sample 1 h after oral administration. Peak identification: 1, osthole; 2, IS.

Moreover, Due to the robustness and the selectivity of the hollow fiber, the method could be applied directly to the determination of analyte without other sample clean-up process, and the repeatability of HF-LPME was also better than that of CPE. The preconcentration effect of HF-LPME is clearly demonstrated in Fig. 6.

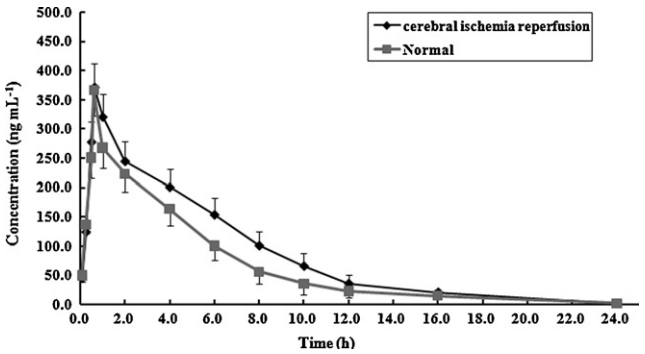

Fig. 8. Plasma concentration–time curve of osthole after oral administration. Each point and bar represents the mean ± SD. Compared with normal rats: [a] $P < 0.05$; [b] $P < 0.01$ ($n = 6$).

Table 5
Pharmacokinetic data of osthole in rat after oral administration ($n = 6$).

Parameter	Estimate (mean ± SD)	
	Normal	Cerebral ischemia hypoperfusion
$T_{1/2}$ (h)	4.94 ± 1.84	8.57 ± 2.52**
T_{max} (h)	0.61 ± 0.09	0.64 ± 0.18
AUC_{0-8} (ng mL^{-1})	0.78 × 10^3 ± 585	1.08 × 10^3 ± 636**
CL (mL kg h^{-1})	0.67 ± 0.15	0.41 ± 0.11*
C_{max} (ng mL^{-1})	366 ± 89	372 ± 76
MRT (h)	5.96 ± 2.71	7.22 ± 3.12*

AUC_{0-8}, the area under curve concentration–time; $T_{1/2}$, half-life time; MRT, mean residence time; CL, clearance.
* Compared with normal rats $P < 0.05$.
** Compared with normal rats $P < 0.01$.

3.4. Pharmacokinetic study of osthole in cerebral ischemia hypoperfusion rat plasma

In our study, a sensitive, economical and accurate HPLC method was developed to determine osthole plasma concentrations in rats after oral administration. The method was used to compare the pharmacokinetics characteristics of osthole in normal rat with that in cerebral ischemia hypoperfusion rats. The results showed significant differences in the main pharmacokinetic parameters of peak time, peak concentration and the area under the concentration–time curve between the two kinds of rat. The mean plasma concentration–time profile is shown in Fig. 8. The pharmacokinetic parameters were calculated and summarized in Table 5. The higher adsorption and lower clearance (CL) of osthole in the cerebral ischemia reperfusion animals may explain the effects of osthole on attenuate cerebral ischemia hypoperfusion injuries.

4. Discussion

In our study, the established method successfully quantified osthole after oral administration and the pharmacokinetic parameters of osthole. Datas showed that there were significant differences in pharmacokinetic parameters in cerebral ischemia hypoperfusion rats and normal rats. It indicated that plasma concentration–time course of osthole in rats was best fitted to a two-compartment open model. After oral administration, the osthole plasma level could be detected after 5 min in both the normal and the cerebral ischemia hypoperfusion model rats, with half-lift ($T_{1/2}$) of 4.94 h in normal animals and 8.57 h in the model animals. Osthole was slowly eliminated from the plasma and was no longer detected after 24 h. We also found that the plasma concentrations of osthole in cerebral ischemia hypoperfusion rats were consistently higher than that in the normal animals. In addition, the cerebral ischemia hypoperfusion model rats had a lower clearance and a longer mean retention time ($P < 0.05$). The reasons for the elimination rate of osthole slowed down in cerebral ischemia hypoperfusion rats may be as follows. First, in the state of pathophysiology, a low activity of certain enzymes and a low ability of biomembral transfer induced by cerebral ischemia hypoperfusion damage might lead to the decreased clearance rate and increased retention time of osthole. Second, osthole is mainly excreted in the urine and the decreased blood circulation of kidney induced by cerebral ischemia hypoperfusion might play an important role in the decreased elimination rate and increased retention time of osthole.

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