Comparative study on brain pharmacokinetics of Buyang Huanwu Decoction in normal and cerebral ischemia rats using brain microdialysis combined with LC-MS/MS

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Abstract

Objective: To conduct a comparative study on the brain pharmacokinetics of seven ingredients (i.e. senkyunolide A, ferulic acid, formononetin, calycosin, ononin, calycosin-O-β-D-glucopyranoside, and paeoniflorin), which were the compounds of Buyang Huanwu Decoction (BHD), in normal and cerebral ischemia rats administrated intragastrically with BHD.

Methods: The samples of normal and permanent middle cerebral artery occlusion (pMCAO) rats were collected by using brain microdialysis technique. The concentrations of seven ingredients were determined by the HPLC-MS/MS method. After the BHD were administrated intragastrically to the rats for seven consecutive days, brain microdialysis probes were inserted into the hippocampus of rats, and then the brain microdialysates were collected at 20 min time intervals for 5 h. The separation of the seven ingredients and internal standard (IS) was carried out on an ACQUITY UPLC BEH C18 (2.1 mm x 100 mm, 1.7 μm) chromatographic column, using a mobile phase consisting of acetonitrile (containing 0.1% formic acid) and water (containing 0.1% formic acid) for gradient elution within 13 min. The ionization was conducted using an ESI source in positive ion mode. Multiple reaction monitoring mode was used for quantification of ingredients in BHD.

Results: Linearity, accuracy, precision, matrix effect and stability of LC-MS/MS method were all satisfactory, successfully applied to compare the pharmacokinetics of the analytes between normal and model rats after intragastric administration of BHD. Compared with the normal group, the model group after the administration of the BHD showed that T1/2 of formononetin and ononin were longer, and except for calycosin-O-β-D-glucopyranoside (P < 0.01), there was no significant difference between the normal group and the model group. The Cmax of senkyunolide A and calycosin of model group were increased, while the Tmax of senkyunolide A was decreased, and except for the Tmax of PF, the differences between the two groups were statistically significant (P < 0.01).

Conclusion: The LC-MS/MS method combined with microdialysis was successfully applied to the comparative study of brain pharmacokinetics of seven ingredients in BHD. After intragastric administration of BHD, there were differences in the pharmacokinetics of seven ingredients in the brain hippocampus between normal rats and model rats, probably related to the characteristics of the ingredients and the effects of cerebral ischemia on the absorption and distribution of the ingredients.

1. Introduction

Cerebral apoplexy, also known as stroke, is a cerebrovascular disease seriously harmful to human health. It has the characteristics of high morbidity, high mortality, high disability rate and high recurrence rate, bringing heavy burden to patients’ family and society. Stroke is divided into ischemic stroke and hemorrhagic stroke, of which ischemic stroke accounts for the majority. At present, the most effective treatment is thrombolytic therapy within 4.5 h of treatment time window. The recombinant tissue plasminogen activator (rt-PA) is the only thrombolytic drug approved by FDA in the United States for the treatment of acute ischemic stroke, but its clinical application is limited by the short treatment window of stroke and its increased risk of hemorrhagic transformation and other side effects (Fu et al., 2014; Gubern-Mérida et al., 2022; Tao et al., 2018). Clinical studies have shown that the first aid treatment of western medicine combined with
the multi-target treatment of traditional Chinese medicine can significantly improve the treatment and prognosis of cerebral ischemia (Chen et al., 2019).

Buyang Huanwu Decoction (BHD) is a classic formula for treating stroke with qi deficiency and blood stasis syndrome, consisting of Astragalus Radix, Angelicae Sinensis Radix, Paeoniae Rubra Radix, Ligustici Chuanxiong Rhizoma, Carthami Flos, Persicae Semen, and Lumbrophus. Studies have demonstrated that BHD play a neuroprotective role in cerebral ischemia injury by alleviating brain edema, reducing cerebral infarction volume and mitigating damage from inflammatory responses, etc. Senkyunolide A (SA), ferulic acid (FA), formononetin (FMN), calycosin (CS), ononin (ON), calycosin-5,7-D-glucopyranoside (CG), paenonolin (PF) are the seven active ingredients contained in BHD. Research has shown that SA can inhibit the release of injury factors after cerebral ischemia/reperfusion (I/R) and relieve inflammation to alleviate neurological deficits (Lin, 2016). FA can inhibit nuclear translocation of NF-κB p65 protein to alleviate the inflammatory injury caused by I/R (Gong, Chen, Zhou, & Zhang, 2019). FMN, CS, ON and CG are four active ingredients in Astragalus Radix, which can induce PC12 cells to promote neurite outgrowth, promote neuronal differentiation, form synapses and establish neural connections, thereby restoring neurological function and protecting blood–brain barrier after cerebral ischemia (Wu, 2020). CS can also treat cerebral I/R injury by improving endothelial cell proliferation and growth (Yu et al., 2021). PF, the active ingredient in Paeoniae Rubra Radix, can reduce the degree of tissue damage in rats with acute cerebral infarction, inhibit the formation of free radicals after cerebral ischemia, and reduce the degree of lipid peroxidation, thereby protecting the ischemic brain (Wu et al., 2020).

Microdialysis technique is mature in brain neurobiology research. Compared with traditional sampling methods, it has the characteristics of “real-time, in vivo, dynamic, minor, microinvasive”. By measuring the target tissue ingredients concentration in microdialysis samples, which can be sampled while the animal is awake, the change information of the ingredients in specific tissues in the real state can be obtained. Since microdialysis samples obtained by dialysis membrane filtration have the characteristics of low concentration, small volume, high salinity and polarity, they require higher requirements for analytical instruments. Liquid chromatography-mass spectrometry has a wide range of analysis, low detection limit, high sensitivity and good tolerance, which is suitable for the detection of microdialysis samples (Zhang, Huang, & Zhu, 2020). Besides, the drying method of the drugs is one of the core links of the whole experiment. Vacuum freeze-drying technology maximizes the appearance quality and active ingredients activity of substance (Xu et al., 2017; Ye et al., 2019), and thus the medicinal value of BHD will be greatly improved by preparing it into lyophilized product before administration.

Pharmacokinetics study under pathological condition is particularly important. Drugs are usually the critical substances that are applied to pathological state and reverse the disorder state of the body. The functional state of the body is an important factor affecting the efficacy of drugs. There may be some differences in the effects of drugs on the body and the disposal of drugs by the body under different functional states. To explore the differences of pharmacokinetics characteristics under different functional states is helpful to provide basis for the treatment of diseases and the rational application of drugs, and is of great significance to clarify the action mechanism. The present study first combined microdialysis sampling technology with LC-MS/MS analysis technology to obtain real and reliable experimental information for the comparative study on the brain pharmacokinetics of seven ingredients in BHD in normal and cerebral ischemia rats. The results would provide reference for further revealing the action mechanism of BHD in the treatment of cerebral ischemia and make it better to apply in clinic.

2. Materials and methods

2.1. Materials and reagents

Reference standards of FMN (lot: wqk19112509, purity: 98%), CS (lot: wqk20010906, purity: 98%), CG (lot: wqk19011506, purity: 98%), PF (lot: wqk16062205, purity: 98%), sulfamethoxazole (IS, lot: wqk20063009, purity: 98%) were purchased from Weikeqi Biotechnology Co., Ltd (Chengdu, China). ON (lot: 19071501, purity: 98%), SA (lot: 17051901, purity: 98%) were purchased from Chengdu Pufei De Biotechnology Co., Ltd (Chengdu, China). FA (lot: E1008544, purity: 98%) was purchased from Macklin Biochemical Co., Ltd (Shanghai, China). HPLC-grade acetonitrile was obtained from Merck (Darmstadt, Germany). HPLC-grade methanol was obtained from Oceanpak (Gothenburg, Sweden). All other reagents were of analytically pure grade. The chemical structures of SA, FA, FMN, CS, ON, CG, PF and sulfamethoxazol (IS) were shown in Fig. 1.

2.2. Preparation of lyophilized products of BHD

The herbal decoction pieces of BHD were mixed with Astragali Radix (lot: 200100961), Angelicae Sinensis Radix (lot: 190304121), Paeoniae Rubra Radix (lot: 190504091), Ligustici Chuanxiong Rhizoma (lot: 19080201), Carthami Flos (lot: 19040371), Persicae Semen (lot: 190700931) and Lumbrophus (lot: 190600641) at a ratio of 120:6:5:3:3:3:3, which were purchased from Kangmei Pharmaceutical Co., Ltd (Puning, China). After being crushed, the herbal admixture was soaked in water for 30 min and was reflushed extraction with five times amount of pure water at 100 °C for 2 h. The extracted solutions were dried by vacuum freeze-drying. The contents of SA, FA, FMN, CS, ON, CG, PF in the lyophilized product of BHD were determined by UPLC-UV, which were 31.53, 63.12, 15.51, 31.06, 102.42, 327.90, 2007.44 μg/g respectively.

2.3. Standard solutions and sample preparations

Artificial cerebrospinal fluid (ACSF), which was used as perfuse for the microdialysis probes, consisted of 144.99 mmol/L sodium chloride, 1.65 mmol/L potassium chloride, 2.14 mmol/L magnesium chloride, 1.59 mmol/L calcium chloride, 0.58 mmol/L sodium dihydrogen phosphate and 3.92 mmol/L dibasic sodium phosphate. ACSF was filtered by a 0.22 µm millipore filter prior to use. Stock solutions of SA, FA, FMN, CS, ON, CG, PF were made by dissolving in methanol, and were diluted gradiently with methanol in order to build the standard curves, the mass concentration: 200, 120, 80, 40, 20, 4 and 2 mg/mL for SA; 1500, 1000, 500, 250, 25 and 5 mg/mL for FA; 500, 300, 200, 100, 50, 10 and 5 mg/mL for FMN; 200, 120, 80, 40, 20, 4 and 2 mg/mL for CS; 300, 150, 60, 30, 15, 3 and 0.6 mg/mL for ON; 200, 120, 80, 40, 20, 4 and 0.4 mg/mL for CG; 600, 400, 200, 100, 20, 10 and 2 mg/mL for PF. QC samples were prepared at concentrations of 200, 40 and 4 mg/mL (SA); 1500, 250 and 25 mg/mL (FA); 500, 100 and 10 mg/mL (FMN); 200, 40 and 4 mg/mL (CS); 300, 60 and 3 mg/mL (ON); 200, 40 and 4 mg/mL (CG); 600, 100 and 10 mg/mL (PF). The IS was diluted to 80 mg/mL by methanol. A total of 2 μL of IS were added to 20 μL of the calibration standard solution, vortex mixing for 5 s. Subsequently, 20 μL of the mixture was injected into the LC–MS/MS system for analysis. The samples were prepared with the same procedure.
2.4. Liquid chromatography-tandem mass spectrometry analysis

2.4.1. Equipment

The LC–MS/MS system was consist of an Agilent 1260 liquid chromatograph, a G1322A online vacuum degasser, a G1312B pump, a G1316A column thermostat, a G1367E autosampler, a G1330B thermostat and a 6460 triple quadrupole mass spectrometry with an electrospray ionization (ESI) source. Data acquisition was performed with MassHunter B.05.00 software.

2.4.2. Chromatographic conditions

Chromatographic separation was performed by an Agilent1260 liquid chromatograph system (Agilent, USA). The seven ingredients and IS were separated on a ACQUITY UPLC BEH C18 (2.1 mm × 100 mm, 1.7 μm, Waters) chromatographic column. The elution gradient for LC–MS/MS analysis consisted of two solvents: water (containing 0.1% formic acid, A) and acetonitrile (containing 0.1% formic acid, B). Gradient elution was carried out according to the following program: 0–2.8 min, 80% → 65% A; 2.8–3.0 min, 65% → 55% A; 3.0–3.5 min, 55% → 40% A; 3.5–4.5 min, 40% → 0% A; 4.5–7.5 min, 0% A; 7.5–8.0 min, 0% → 10% A; 8.0–8.5 min, 10% → 30% A; 8.5–9.0 min, 30% → 60% A; 9–10 min, 60% → 70% A; 10–13 min, 70% → 80% A. The initial 3.6 min was switched to the waste channel. The column temperature at 30 °C and the injection volume was 20 μL.

2.4.3. Mass spectrometric conditions

A 6460 triple quadrupole tandem mass spectrometer (Agilent, USA) with electrospray source was operated in positive ion mode. Data acquisition was performed with MassHunter B.05.00 software.

2.5. Method validation

Validation of the proposed method was performed with respect to selectivity, linearity, accuracy and precision, matrix effect and stability.

2.5.1. Selectivity

Selectivity was tested by comparison of blank ACSF, mixed standard solution and the microdialysis sample, investigating whether endogenous dialysate matrix ingredients had potential interferences with retention time for all analytes and IS.

2.5.2. Linearity, LOD and LOQ

The linearity of the method was evaluated by testing each concentration level of the seven calibration standards in duplicate. The calibration curves were constructed by plotting the peak area ratio (S/N) of the analyte to IS versus the concentrations of the analyte (x). The linearity of each calibration curve was defined by a correlation coefficient (r) of at least 0.995. The limit of detection (LOD) was defined as the concentration when the signal-to-noise ratio (S/N) was 3, and the limit of quantity (LOQ) was defined as the concentration when the S/N was 10.

2.5.3. Precision and accuracy

Precision was determined by analyzing QC samples at three concentration levels in six replicates. Precision was evaluated by relative standard deviation (RSD = [standard deviation (SD)/Cobs]× 100%). Accuracy was calculated as (Cobs/Cactual) × 100%. The value of precision was required to be no more than 15%, and accuracy was required to be within 85–115%.

2.5.4. Matrix effects

The matrix effects for the seven ingredients and IS were assessed by assaying six replicates of QCs at three concentration levels. This was assessed by comparing the peak area ratios (y) of the analyte to IS versus the concentrations of the analyte (x). The matrix effects was calculated as (AACSF/Amethanol) × 100%. The value of matrix effects was required to be within 85–115%.

2.5.5. Stability

The stability of the seven ingredients was evaluated by analyzing QCs at three concentration levels at room temperature for 24 h. These QCs were analyzed after storage at room temperature at 0, 2, 4, 6, 8, 12, 24 h. Stability were evaluated by RSD. The value of stability was required to be no more than 15%.
2.6. Animals and surgery procedure

Male Sprague-Dawley rats, weighed (300 ± 20) g, were supplied by the Laboratory Animal Center of Guangzhou University of Chinese Medicine (approval number: SCXK Yue 2018-0034) and raised in the specific pathogen free room of Laboratory Animal Center of Guangzhou University of Chinese Medicine (approval number: SYXX Yue 2018-0085). Rats were given free access to food and water and were raised under controlled temperature [25 ± 1 °C] and humidity (55%–70%) with a 12 h light dark cycle. All experiments were performed in accordance with the Guide of National Institutes of Health and approved by the Animal Care and Use Committee of the Guangzhou University of Chinese Medicine (Guangdong, China). After 3 d of adaptive housing and feeding, the rats were randomly divided into normal group and model group (with six rats in each group). Rats were fasted for 12 h before surgery but provided adequate water supply.

2.6.1. Permanent middle cerebral artery occlusion (pMCAO) surgery

The pMCAO model was established according to the method of Longa with minor modifications to induce cerebral ischemia of rats in model group. Briefly, the rats were anesthetized with 10% chloral hydrate (350 mg/kg, ip) and their body temperature were maintained 37 °C by a heating blanket. After disinfection of the neck region, a midline neck incision was performed and then the right common carotid artery (CCA), the external carotid artery (ECA), and the internal carotid artery (ICA) were isolated, and the ECA and CCA were ligated. A 0.26-mm silica gel-coated nylon monofilament was inserted through the ICA, 18–20 mm from the carotid bifurcation, to occlude the the middle cerebral artery (MCA) in the brain. 2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) staining and neurological scores determined whether the pMCAO model was successful. After decapitation of the rat, its brain was sliced and stained with TTC. Red colored region in the TTC stained sections indicates non-ischemic and pale colored region indicates ischemic portion of brain (Xiao et al., 2010). The neurological deficit scores were performed after 24 h of the pMCAO surgery in model group. The neurologic findings were scored by the method of Longa on a five-point scale: A score of 0 indicated no neurologic deficit, a score of 1 (failure to extend left forepaw fully) a mild focal neurologic deficit, a score of 2 (circling to the left) a moderate focal neurologic deficit, and a score of 3 (falling to the left) a severe focal deficit; Rats with a score of 4 did not walk spontaneously and had a depressed level of consciousness (Longa, Weinstein, Carlson, & Cummins, 1989). Rats of a score of 1 or 2 would be used for subsequent experiments. Intragastric administration of BHD to the rats for seven consecutive days starting on the second day of pMCAO surgery.

2.6.2. Microdialysis cannula implantation surgery

On the 6th day after modeling, the rats were anesthetized, and their heads were shaved before placing them in a stereotaxic apparatus (RWD, Shenzhen, China). An incision was made in the scalp. The bregma was located and used as the reference point for positioning the microdialysis probe. A microdialysis guide cannula (CMA/Microdialysis AB, Stockholm, Sweden) was stereotaxically inserted in a cranial burr hole made by a skull drill using the following coordinates, in relation to the bregma (hippocampus: AP –5.3 mm, ML +5.0 mm, DV –4.5 mm). Two small rivets were screwed near the hole to immobilize the microdialysis guide cannula. After the microdialysis guide cannula was implanted, the glass ionomer cements were poured on the cathead and rivets. Subsequently, one day after the surgery, the rats were ready for sampling. After the sampling, the rats were decapitated and their brains were taken, and the hippocampus was separated to verify the probe implantation position.

2.7. Drug administration and brain pharmacokinetics study

On the seventh day after modeling, the guide cannula was replaced by the microdialysis probes with 4 mm membranes which were purchased from CMA/Microdialysis AB (Stockholm, Sweden). The brain microdialysis probes were perfused with ACSF at 2.0 μL/min. The rats were administrated the last dose of BHD intragastrically after a 1 h equilibration time. The microdialysis samples were collected every 20 min for 5 h. The collected samples were kept at –80 °C until analyzed.

2.8. Determination of recovery of seven ingredients in BHD

To obtain an accurate quantification of the analyte, the microdialysis probe should be calibrated by determining its recovery using a retrodialysis method. After the 5 h sampling, the brain microdialysis probe was not removed, and ACSF perfusion was continued for 2 h. Then, ACSF was replaced with 100 ng/mL of the seven-ingredient mixed standard dissolved by ACSF. The microdialysis samples were collected every 20 min for 5 h at the flow velocity of 2.0 μL/min for the use of recovery correction. The drug concentration was calculated and the relative loss rate (RL) in the body was calculated by the formula $RL = \left( \frac{C_{\text{perfusate}} - C_{\text{dialysate}}}{{C_{\text{perfusate}}} \times 100}\right)$, and the actual concentration of the drug was calculated by the formula $C_{\text{actual}} = \frac{C_{\text{dialysate}}}{RL}$.

2.9. Data analysis

Concentration-time curves were obtained by plotting the midpoint of time as the abscissa and the microdialysis sample concentration as the ordinate. Pharmacokinetics parameters were calculated using a noncompartmental model by WinNonlin 4.0.1 software (Pharsight Co., USA) and were represented as mean ± standard deviation (SD). Statistical analysis comparisons of the two groups were performed by SPSS 20.0 (IBM, USA) software. If the data conform to the normal distribution, the independent sample t test method was used for statistical analysis; if the data did not conform to the normal distribution, the nonparametric test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Method development

In present study, the response values of the seven ingredients in positive and negative ion modes were investigated. Though the response values in the negative ion mode of FMN and CS were higher than those in the positive ion mode, the others were not. Therefore, the positive ion mode was finally selected for the simultaneous determination of seven ingredients and IS. The MS parameters, such as fragmentor and collision energy were optimized to obtain the higher signal for precursor ions and product ions. The MRM transitions selected to analyze SA, FA, FMN, CS, ON, CG, PF and IS were summarized in Table 1. The chromatographic conditions were investigated to optimize resolution and peak shape. Methanol-water and acetonitrile-water were successively used as mobile phases. Methanol would prolong the overall retention time of the seven ingredients and IS, which was not conducive to the detection efficiency. When acetonitrile was used as organic phase, each ingredient to be measured could peak before 13 min. The peak shapes and response values of each ingredient were comprehensively compared after adding 5 mmol/L ammonium formate, 5 mmol/L ammonium acetate and 0.1% formic acid. Finally, acetonitrile (containing 0.1% formic acid)-water (containing 0.1%
formic acid) was selected as the mobile phase. The peak shapes of each tested ingredient were fine, without interference of endogenous substances.

3.2. Method validation

In the methodological validation research, a comprehensive investigation on selectivity, linearity and sensitivity, precision and accuracy, matrix effects and stability of method were carried out. All the results of methodological validation demonstrated that the method was reliable, stable and suitable for the present study.

3.2.1. Selectivity

Fig. 2 demonstrated typical chromatograms of the blank ACSF (A), mixed standard solution (B) and the microdialysis sample obtained after administration of BHD to the rats (C). No significant interference by endogenous substance was observed under the established condition, indicating a well selectivity. The running time of each injection was 13 min. The retention time of SA, FA, FMN, CS, ON, CG, PF, and IS were 11.3, 7.5, 10.4, 9.8, 9.4, 5.5, 5.2 and 9.3 min, respectively.

3.2.2. Linearity and sensitivity

Table 2 showed the regression equations of the calibration curve, and the \( r \) values were all higher than 0.9954, indicating excellent linearity. The LOD and LOQ of seven ingredients in BHD were sufficient for the brain pharmacokinetics study in rats administrated intragastrically with the lyophilized product of BHD.

3.2.3. Precision and accuracy

Table 3 summarized the precisions and accuracies of seven ingredients at three concentration levels. The RSD of the accuracies were lower than 4.29% and the precision ranged from 85.22% to 114.55%, which were well within the acceptance criteria of bioanalysis and indicated that the method was accurate, reliable, and reproducible.
3.2.4. Matrix effects

The occurrence of matrix effects was associated with the complexity of the samples, which would cause either ion suppression or enhancement of the signal. Cerebrospinal fluid is mainly composed of electrolyte. In the established method, a relatively high proportion of aqueous phase was used for elution in the first 2.8 min, so that the salt contained in the matrix was eluted first. The eluent is cut into the waste channel for the first 3.6 min, and then divert into the mass spectrometer for detection and analysis after 3.6 min, which could not only reduce the matrix effect, but also avoid mass spectrometer contaminated by electrolyte. As seen in Table 3, the matrix effects at three concentrations of each ingre-

Table 3

| Ingredients                        | Concentration (ng/mL) | Precision (%) | Accuracy (%) | Matrix effect (%) | Stability |
|------------------------------------|-----------------------|---------------|--------------|-------------------|-----------|
| Senkyunolide A                     | 4                     | 0.13          | 114.55       | 100.14            | 8.28      |
|                                    | 40                    | 0.90          | 85.22        | 113.40            | 4.35      |
|                                    | 200                   | 0.41          | 112.55       | 89.27             | 3.32      |
| Ferulic acid                       | 25                    | 0.08          | 114.51       | 97.21             | 10.47     |
|                                    | 250                   | 0.55          | 93.33        | 114.64            | 3.06      |
|                                    | 1500                  | 0.77          | 92.31        | 102.37            | 3.34      |
| Formononetin                       | 10                    | 0.08          | 109.88       | 98.27             | 10.74     |
|                                    | 100                   | 0.28          | 85.56        | 114.36            | 10.81     |
|                                    | 500                   | 0.37          | 98.82        | 99.26             | 6.67      |
| Calycosin                          | 4                     | 0.40          | 110.88       | 98.68             | 7.23      |
|                                    | 40                    | 0.42          | 112.19       | 92.56             | 2.51      |
|                                    | 200                   | 1.84          | 109.93       | 87.08             | 4.75      |
| Ononin                             | 3                     | 0.11          | 87.92        | 88.67             | 1.40      |
|                                    | 60                    | 0.65          | 91.28        | 85.17             | 1.09      |
|                                    | 300                   | 1.00          | 102.23       | 104.42            | 1.50      |
| Calycosin-O-β-D-glucopyranoside    | 4                     | 0.30          | 105.76       | 94.01             | 3.22      |
|                                    | 40                    | 1.08          | 114.08       | 90.74             | 1.16      |
|                                    | 200                   | 1.71          | 102.89       | 112.62            | 1.66      |
| Paeoniflorin                       | 10                    | 0.10          | 85.97        | 94.08             | 5.54      |
|                                    | 100                   | 0.47          | 114.34       | 98.75             | 5.68      |
|                                    | 600                   | 4.29          | 106.27       | 91.31             | 7.01      |

Fig. 3. Brain microdialysate samples concentration–time curves of seven ingredients (A: senkyunolide A; B: ferulic acid; C: formononetin; D: calycosin; E: ononin; F: calycosin-O-β-D-glucopyranoside; G: paeoniflorin) in normal and model groups.
dient were between 85.17% and 114.64%, demonstrating that the matrix effects were within the acceptable range.

### 3.2.5. Stability

As seen in Table 3, the stability results showed that the RSD of stabilities were lower than 10.81%, demonstrating the method had a good stability within 24 h.

### 3.3. Recovery of microdialysis probe

The averages of in vivo recoveries of SA, FA, FMN, CS, ON, CG and PF in hippocampus were 7.84%, 46.63%, 38.54%, 21.48%, 89.14% and 21.48%, respectively. The concentrations of samples were converted according to the probe recoveries before calculating the pharmacokinetics data.

### 3.4. Results and discussion of comparative study on brain pharmacokinetics of BHD in normal/pMCAO rats

The developed LC–MS/MS method was applied to investigate the brain pharmacokinetics of the seven ingredients in normal and model rats after ig administration of BHD. The rats in the normal group had no symptoms of neurological deficit, and the score was 0, and there was no pale infarct area after TTC staining. The rats in the model group showed symptoms of neurological deficit, and the score was 1–2, and the affected side of the brain after TTC staining appeared pale infarct area, indicating that the pMCAO model was successfully established with good repeatability. The mean brain concentration–time curves of seven ingredients in normal and model rats were illustrated in Fig. 3 and the estimated pharmacokinetics parameters were listed in Table 4. Compared with the normal group, the model group after the administration of the BHD showed that T1/2 of FMN and ON were longer, and T1/2 and MRT of FMN were bigger than PF, which was consistent with the results of the present experiment. The AUC0–t of FMN was less than PF (Wang et al., 2020), which was different from the results of the present experiment. The difference may be caused by the different modeling method and intragastric administration dosage of BHD.

In the present study, the results indicated that cerebral ischemic injury may change the permeability of blood–brain barrier, and affect the digestion, absorption and metabolism of drugs in vivo, which affect the peak time, peak concentration and residence time of ingredients in brain. Studies have shown that cerebral ischemia brings great harm to the physical and mental health of patients, and can lead to a series of complications, such as swallowing disorders, malnutrition, renal insufficiency, hypertension, depression, digestive tract diseases, etc. (Lin, Su, Yu, Luo, & Chen, 2021). Among the complications, gastrointestinal dysfunction, such as constipation, abdominal distension and gastrointestinal...
nal bleeding, which have a high incidence in stroke patients (Liu, & Wei, 2020), may also be the key factor for the prolonged peak time and low concentration of most ingredients in the model rats, in addition to the influence of the change of blood–brain barrier permeability.

4. Conclusion

In conclusion, normal rats and cerebral ischemia model rats have significant differences in BHD brain pharmacokinetics. Seven ingredients in BHD can enter the brain and play the neuroprotective role. Cerebral ischemia injury may change the permeability of blood–brain barrier. Complications such as digestive tract injury caused by cerebral ischemia may affect the pharmacokinetics process of the ingredients.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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