

Full Length Research Paper

***In-vivo* studies on the pharmacokinetics of berberine on middle cerebral artery occlusion rat and sham-operated rat**

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The present study aims to investigate the influence of cerebrovascular disease and herb prescription on the pharmacokinetics of berberine. In the designed study, rats were divided into middle cerebral artery occlusion (MCAO) group and sham-operated group. Each group contained two subgroups: Oral administration of Huang-Lian-Jie-Du-Tang (HLJDT) (is equal to 258 mg berberine/kg body weight) and pure berberine (is equal to 258 mg berberine/kg body weight, too). The pharmacokinetics of berberine following oral administration of pure berberine or Huang-Lian-Jie-Du-Tang (HLJDT) investigated in MCAO and sham-operated rats were compared. The pharmacokinetics results indicated that both in the two groups, the AUC and C_{max} of berberine in HLJDT group were larger than in pure berberine group. Besides, the absorption of berberine in MCAO group was obviously better and the metabolic rate of berberine was slower than in sham-operated group. The authors inferred that the cerebrovascular disease and prescription of HLJDT has increased the bioavailability of berberine *in-vivo*. It noticed that the better absorption of berberine would be in favor of berberine to exert pharmacological effects on cerebrovascular disease. In this sense, it is necessary to take the pharmacokinetics characteristics of berberine in different conditions into consideration in clinics. This study may give an advice for further herbal research which will be carried on the same subject.

Key words: Huang-Lian-Jie-Du-Tang, berberine, pharmacokinetics, middle cerebral artery occlusion (MCAO), compatibility

INTRODUCTION

Huang-Lian-Jie-Du-Tang (HLJDT) consists of *Rhizome coptidis*, *Radix scutellariae*, *Cortex phellodendri* and *Fructus gardenia*, with the ratio of 3:2:2:3. It has been used for more than two thousand years by Chinese people for treatment of cerebral diseases in china

(Kabuto et al., 1997). Even today HLJDT has been used for cerebrovascular disease in clinic with good application too (Itoh, 2001; Fujiwara and Iwasaki, 1993). It was observed that HLJDT could reduce ischemia-reperfusion brain injury in rats (Hwang et al., 2002). Furthermore, a protective effect of Oren-gedoku-to (Huang-Lian-Jie-Du-Tang) against impairment of learning and memory induced by transient cerebral ischemia in mice has been reported (Xu et al., 2000).

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Abbreviations: HLJDT, Huang-Lian-Jie-Du-Tang; MCAO, middle cerebral artery occlusion; IS, internal standard; AUC, area under the curve; MRT, mean residence time; A.R, analytical-reagent.

Berberine, an isoquinoline alkaloid, is one of main components in *R. coptidis* and *C. phellodendri*, which are the key ingredient herbs in HLJDT. Pharmacological studies have indicated that berberine shares many bioactivities with respect to antibiotic, antiinflammatory, antidiarrheal, antineoplastic and so on (Tang et al., 2009).

Table 1. Sources of chemicals and experimental materials used in the experiment.

Name	Purity	Company
<i>Rhizome coptidis</i> (Rhizome)		Bozhou Medicine Company (Anhui, China)
<i>Radix scutellariae</i> (Root)		Bozhou Medicine Company (Anhui, China)
<i>Cortex phellodendri</i> (Cortex)		Bozhou Medicine Company (Anhui, China)
<i>Fructus gardenia</i> (Fruit)		Bozhou Medicine Company (Anhui, China)
pure berberine	≥95%	Zelang Biotechnology Company (Nanjing, China)
B berberine	≥98%	National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China)
Ps psoralen	≥98%	National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China)
Acetonitrile	HPLC	Merck Company (New Jersey, USA)
Methanol	HPLC	Han Bang Company (Jiangsu, China)
Deionized water	HPLC	Wahaha Company (Hangzhou, China)
Phosphoric acid	A.R.	Jiuyi chemical Reagent Company (Shanghai, China)
Sodium dodecyl sulfates (SDS)	A.R.	Jiuyi chemical Reagent Company (Shanghai, China)
Potassiumdihydrogen phosphate(KDP)	A.R.	Jiuyi chemical Reagent Company (Shanghai, China)

Moreover, a lot of researchers have reported the protective effects of berberine and *R. coptidis* on rats against cerebral ischemia (Wei et al., 1995; Wu et al., 1995; Xi et al., 2001,2003; Zhou et al., 2008; Cho et al., 2004).

Several investigators have reported the qualitative and quantitative analysis of berberine in plasma, liver, bile, urine, hippocampus of rats, mice and humans by high performance liquid chromatograph-ultraviolet (HPLC-UV), high performance liquid chromatograph mass spectrometer (HPLC-MS), high performance liquid chromatography-electrospray tandem mass spectrometry (HPLC-ESI-MS/MS) (Tsai and Thai, 2002; Lu et al., 2006; Wang et al., 2005; Chen and Chang, 1995; Hua et al., 2007; Deng et al., 2008; Gupta et al., 2009). However, little information focuses on whether the pharmacokinetics of berberine in pathological model would be affected by herbs interaction or cerebrovascular disease. To the best of our knowledge, the effect of medicine for the therapy of disease is usually based on the information given by certain disease. As both berberine and HLJDT have beneficial effects on cerebrovascular disease, the opportunity to compare them in pathological model would seem attractive. A major concern is that an herb interaction that would affect the pharmacokinetics of berberine may occur. And to understand pharmacokinetics behavior of berberine *in-vivo* seems very necessary. Based on the aforementioned, the purpose of this paper is to analyze the rationality of the compatibility of HLJDT and the effect of cerebrovascular disease to the pharmacokinetics of berberine. In order to improve the safety of herbal application in clinic, it requires more necessity to establish an effective, valid and convenient detection

method to research the pharmacokinetics of berberine.

MATERIALS AND METHODS

Materials

Sources of chemicals and experimental materials which are used in the experiment are shown in the Table 1.

R. coptidis (Rhizome), *R. scutellariae* (Root), *C. phellodendri* (Cortex) and *F. gardenia* (Fruit) was identified by professor Qi-Nan Wu (Department of pharmacognosy, Nanjing University of Chinese Medicine, Nanjing, China). The voucher specimens were deposited in Nanjing University of Chinese Medicine.

The structures of berberine reference standard and internal standard (IS) psoralen are shown in Figure 1

HPLC system

The HPLC system consisted of a Waters 1525 pump, 2487 dual λ absorbance detector, and injector with a 10- μ l loop. Data were processed using empower workstation (Zhejiang, China). Chromatographic separation was performed on an Agilent C₁₈ column (250 × 4.6 mm i.d., particle size 5 μ m, USA). The mobile phase was acetonitrile-0.1% phosphoric acid solution (50:50, v/v, every 100 ml contains 0.1 g SDS), and the flow rate of the mobile phase was 1.0 ml/min. Detecting ultraviolet (UV) wavelength was set at 340 nm, with a sensitivity setting of 0.05 AUFS. The injection volume was 5 μ l and the column maintained at a constant temperature 30°C.

Preparation of oral medicine

30 g of *R. coptidis* (Rhizome), 20 g of *R. scutellariae* (Root) 20 g of *C. phellodendri* (Cortex), and 30 g of *F. gardenia* (Fruit) were extracted twice by refluxing with boiling water (1:10, and 1:8, w/v) for 1 h each time. The HLJDT extract was spray-dried. The yield of HLJDT extract was 25.2% (w/w) in terms of the dried medicinal

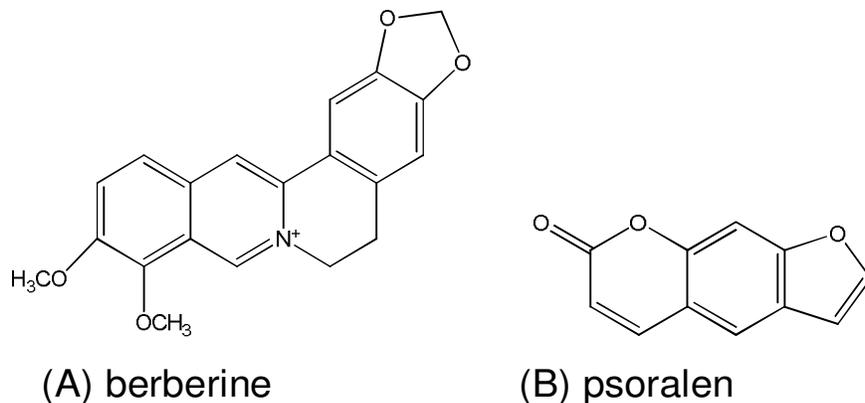


Figure 1. Structure of berberine and IS (psoralen).

herbs. The dried powder was stored at 4°C before use. The concentration of berberine in HLJDT extract and pure berberine were analyzed by HPLC. 0.10 g HLJDT extract was ultrasound with 50 ml of methanol for 30 min and then the solution passed through a 0.45- μ m microporous membrane before HPLC analysis. The content of berberine was 2.58 g/100 g HLJDT extract and 952.5 mg/g pure berberine, respectively.

Animals

Male Sprague Dawley (SD) rats (280 to 300 g) were obtained from the Laboratory Animal Center of the Nanjing University of Chinese Medicine, Jiangsu, China. Animal welfare and experimental procedures were strictly in accordance with the Guide for the Care and Use of Laboratory Animals (US National Research Council, 1996). Preparation of MCAO and sham-operated rats Animals was anesthetized with 10% chloral hydrate solution. The middle cerebral artery (MCA) was occluded according to the method of Nagasawa and Kogure (1989), with minor modifications. Briefly, on rat was made a median incision in the right head skin to expose the middle cerebral artery (MCA). Blood vessels were separated to make them free and electric knife was then used to electric coagulate the part of middle cerebral artery, which was from olfactory tract to the brain lower vein. The MCA was blocked and the wound then stitched. The same approach was used for the sham-operated rats except electric coagulation. 4 h after surgery, as a successful replication of the model of MCAO, the body temperature will rise over 0.8°C, and rats show visible symptoms of neurological deficit characterized by severe left-sided hemiparesis and right Horner's syndrome (Longa et al., 1989; Bederson et al., 1986). It is mainly shown in activities' reduction, apathy, and dumping to the right side when walking. If it keeps on rotating, the neurobiology score significantly increased. These were criteria for evaluating the ischemic insult. Rats, which did not show behavioral deficit were excluded from the MCAO group, and then divided into two subgroups randomly. The sham-operated rats were divided into two subgroups randomly too. The rats had free access to food and water until 12 h prior to being used in experiments.

Calibration curves and quality control samples

The berberine reference standard and internal standard (IS) psoralen were accurately weighted and dissolved in methanol to obtain matrix standard. The matrix standard of berberine and IS at the concentration of 54.4 μ g/ml and 30 μ g/ml for each was prepared

respectively. Then the matrix standard of berberine was diluted to seven different concentrations berberine standard solutions at 0.085, 0.17, 0.34, 0.68, 1.36, 2.72 and 5.44 μ g/ml. A 30 μ g/ml stock solution of IS was also prepared in methanol and then diluted to 1.5 μ g/ml. The solution was stored at 4°C before use.

The calibration curves samples were prepared by spiking the 100 μ l blank rat plasma with 100 μ l of the berberine standard solutions of 0.085, 0.17, 0.34, 0.68, 1.36, 2.72 and 5.44 μ g/ml, respectively, containing 1.5 μ g/ml psoralen as the inter standard.

The analytical standard and quality control samples were prepared at the concentration of 0.17, 0.68 and 2.72 μ g/ml.

Method validation

Selectivity

Assay selectivity was evaluated by analyzing blank plasma samples. The chromatogram of blank plasma sample was compared with those of samples at the concentration of LLOQ which had response more than blank plasma. Blank plasma samples was spiked with IS (1.5 μ g/ml) to analyzed the interference of IS.

Calibration curves of berberine

The calibration curves samples had been prepared. Calibration curves were established by determining peak areas, using unweighted linear regression of the peak area ration of berberine to IS(Y) against the corresponding spiked plasma concentrations of the berberine (X).

Recovery and precision

The recovery and precision (both intra- and inter- day) were determined by analyzing berberine at the concentration of high, medium, low of rat plasma was 0.17, 0.68 and 2.72 μ g/ml, respectively. The recovery and precision (both intra- and inter- day) samples were prepared from 100 μ l blank rat plasma spiking with the berberine standard solutions and IS solution. The concentration of each sample was calculated using a calibration curve on the same testing day. The recovery of the berberine was determined by comparing the mean peak areas of six extracted low, medium, high samples to mean peak areas of low, medium, high berberine standard solution. The recovery of IS was calculated by the same

way. The intra- and inter- day precision was determined by analyzing berberine at the concentration of high, medium, low of rat plasma five times on the same day and continuously for 5 days, respectively.

Stability

The stability of berberine evaluated for high, medium, low of rat plasma was 0.17, 0.68 and 2.72 $\mu\text{g/ml}$, respectively. Under conditions mimicking situations likely to be encountered during sample storage and the analytical process was analyzing five replicates of QC (quality control) samples. The freeze-thaw stability was determined after one freeze and thaw cycle. The QC samples were stored at -20°C for 24 h and thawed unassisted at room temperature. The average peak areas of berberine in the samples and RSD (relative standard deviation) were calculated.

Sample preparation

The 100 μl of rat plasma in a 2-ml centrifuge tube, 100 μl of 1.5 $\mu\text{g/ml}$ of IS, 50 μl of 1 mol/l K_2HPO_4 aqueous solution and 400 μl of methanol were added. The mixture was vortex-mixed for 30 s and centrifuged for 5 min at 5000 rpm. Then the supernatant was transferred to clean centrifuge tube and evaporated to dryness under a stream of nitrogen at 40°C water bath. The residues were reconstituted in 100 μl methanol followed by vortex mixing for 30 s. Finally, samples were centrifuged for 5 min at 5000 rpm; the supernatant was transferred to a clean centrifuge tube. A 5 μl aliquot of the supernatant was injected into the HPLC system for analysis.

Application to pharmacokinetics study

The HLJDT extract powder was dissolved in 0.50% carboxymethyl cellulose sodium (CMC-Na) aqueous solution and was administered to the rats extract powder 10 g/kg body weight, containing 258 mg berberine/kg body weight by oral gavage. The pure berberine was administered by the same way at a dose of 2.7 g/kg body weight, containing 258 mg berberine/kg body weight too. Blood samples (500 μl) were taken from the abdominal vein before dosing (to serve as a blank) and subsequently at 0.083, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 12.0 and 24.0 h following medicine administration. Blood samples were transferred to heparinized centrifuge tube and centrifuged at 5000 rpm for 5 min to separate out plasma. The plasma was transferred to clean centrifuge tube and stored at -20°C until analysis.

Data analysis

The concentrations of berberine in the rat plasma were determined from the calibration. Data were expressed as mean \pm SD. The concentration-time data were analyzed by non-compartmental methods using the DAS 2.0 (Mathematical Pharmacology Professional Committee of China, Shanghai, China) (Chen et al., 2002).

RESULTS AND DISCUSSION

Calibration curve

The linearity of the calibration curve was evaluated by

analysis of peak area ratios (berberine/IS) to the berberine concentrations spiked into blank rat plasma. The calibration curve for the berberine was linear $r^2 = 0.9994$, over the concentration range of 0.085 to 5.44 $\mu\text{g/ml}$ in rat plasma. The typical regression equation for the calibration curve of rat plasma was $Y = 0.292X + 0.046$ (X = concentration of berberine spiked in blank rat plasma; Y = the peak area ratio of berberine/IS). The LLOQ (lower limit of quantitation) was established at 0.085 $\mu\text{g/ml}$.

Method validation

Selectivity

As shown in Figure 2, no significant interfering peaks were observed in the blank plasma. The retention times for berberine and IS were 7.973 and 11.193 min, respectively.

Recovery and precision

The recoveries of berberine were shown in the Table 1. The recoveries of the analytes were shown to be consistent and reproducible. The intra and inter-day precision of berberine were summarized in Table 2. The results demonstrate that the precision of this assay were acceptable.

Stability

The stability of analyte was investigated at three concentration levels (0.17, 0.68 and 2.72 $\mu\text{g/ml}$). Berberine shown good stability was stored at -20°C for 24 h and thawed unassisted at room temperature for one week; the concentration of berberine in plasma deviated to less than $\pm 8\%$ from those in freshly spiked plasma.

Results of pharmacokinetics study

After oral administration of HLJDT extract or pure berberine to rats, the concentration of berberine in rat plasma was determined by HPLC system. The plasma concentration-time profile for berberine was represented in Figure 3. The pharmacokinetics parameters were listed in Table 3. The concentration of berberine in plasma rose rapidly, but the amount of berberine entering the blood circulation was very low. There were two absorption peaks observed in the concentration-time curves of berberine. Besides, the AUC and maximum concentration (C_{max}) of berberine in HLJDT extract groups were larger than in the pure berberine groups. The phenomenon occurred both in MCAO and sham-

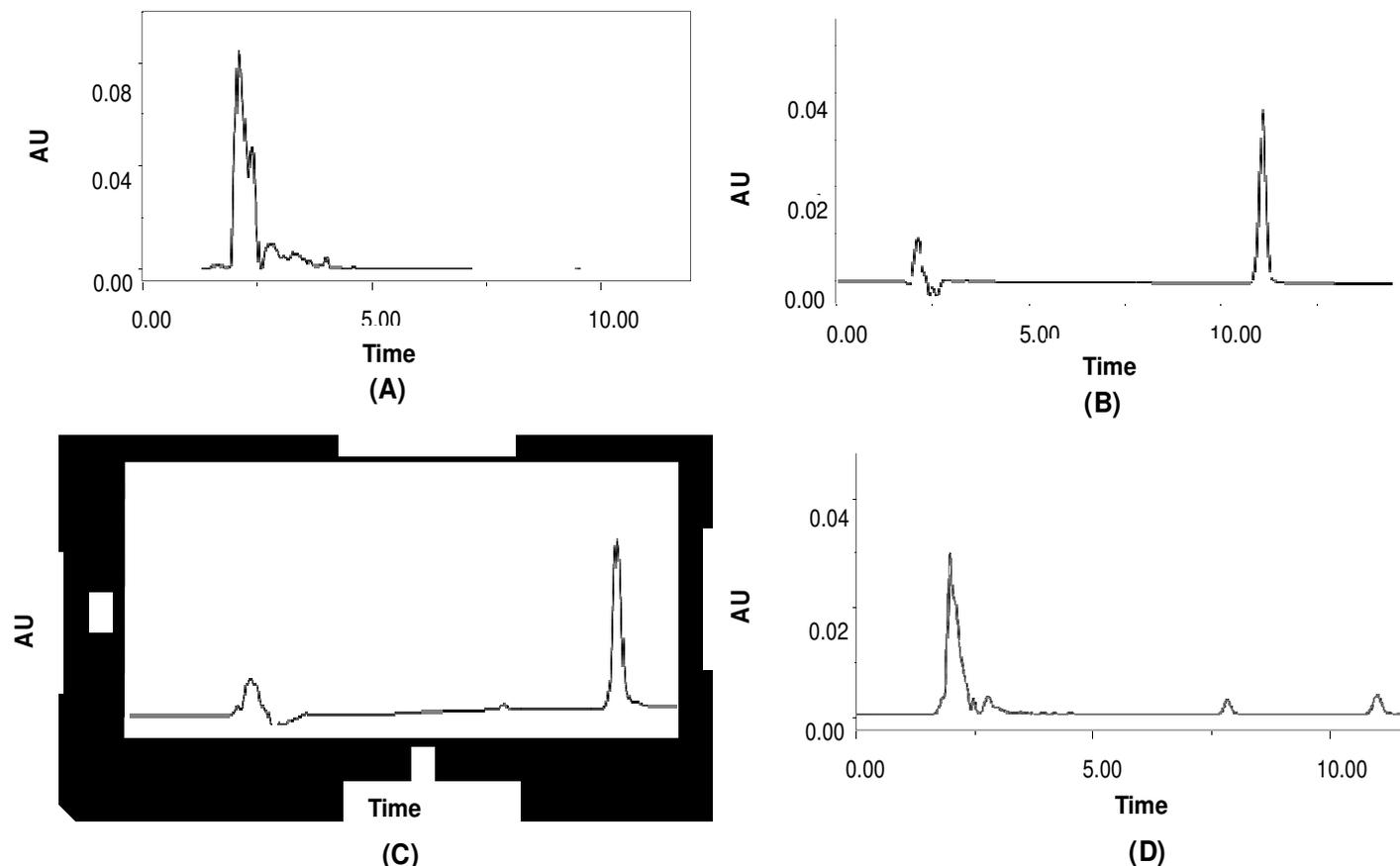


Figure 2. Typical chromatogram for injection of (A) blank plasma, (B) standard berberine, (C) standard IS, and (D) a plasma sample collected from rat after 1 h oral administration of HLJDT.

Table 2. The recovery of berberine in plasma.

Spiked concentration ($\mu\text{g/ml}$)	Measured concentration ($\mu\text{g/ml}$)	Recovery (%)
0.17	0.14 ± 0.01	79.98 ± 4.72
0.68	0.59 ± 0.03	85.97 ± 4.27
2.72	2.43 ± 0.15	89.28 ± 5.42

operated groups. At the same time, the absorption of berberine in MCAO group was obviously larger and metabolized slower than in the sham-operated group. In addition, in MCAO group the second absorption peak of berberine was larger than the sham-operated group, too.

Discussion

Based on the results, the authors analyzed the pharmacokinetics characteristic of berberine and their differences among subgroups. When analyzing the

pharmacokinetics of berberine in different groups, the author found that there were two peaks in the plasma concentration-time curves, berberine absorbed quickly *in vivo*; the T_{max} was 45 min approximately. The results of the study were in accord with references. Deng et al. (2008) had reported that there were three absorption peaks after oral administration of coptis-evodia herb couple and the T_{max} was 90 min. Wu et al. (2009) had studied the pharmacokinetics of berberine after oral administration of HLJDT extract; it presented two absorption peaks, and the T_{max} was 60 min approximately. The reason for the multi absorption peaks

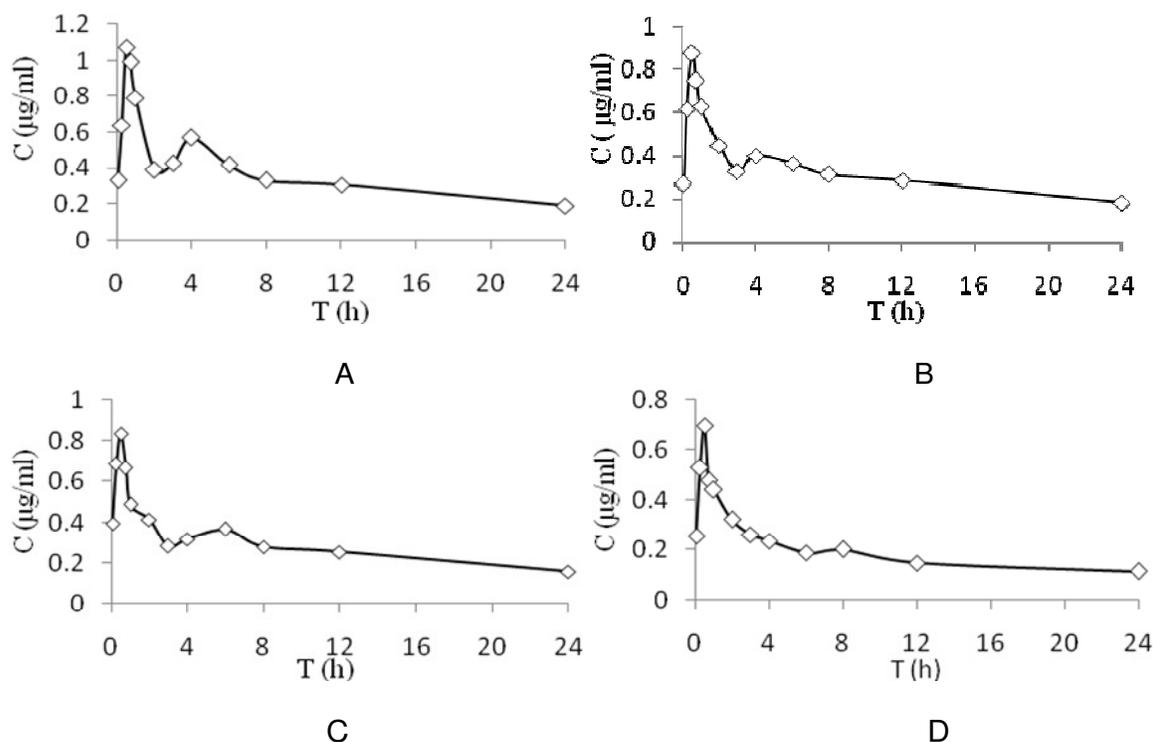


Figure 3. Mean plasma concentration (C)-time (T) curves of berberine after oral administration of HLJDT decoction or pure berberine (258 mg berberine/kg body weight) (A) MCAO rats oral of HLJDT extract; (B) MCAO rats oral of pure berberine; (C) Sham-operated rats oral of HLJDT extract; (D) Sham-operated oral of pure berberine.

Table 3. Intra- and inter-day precision of berberine.

Concentration ($\mu\text{g/ml}$)	RSD (%)		RE (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
0.17	4.73	6.93	-14.97	-5.52
0.68	2.65	2.65	1.92	1.13
2.72	1.17	3.38	3.26	8.83

RE, Release error.

of berberine in plasma maybe contributed to the distribution re-absorption and enterohepatic circulation.

On the other hand, the absorption of berberine *in-vivo* was influenced by other medicine, such as Li et al. (2000) reported Oryzanol could do well to the absorption of berberine. Wang et al. (2007) presented that *R. Coptidis* combination with *R. Rehmannia* could effectively enhance the bioavailability of berberine in rats. The authors compared the different pharmacokinetics characteristics of berberine after oral administration of HLJDT extract or pure berberine to evaluate the rationality of the compatibility of HLJDT. The AUC and C_{max} in HLJDT groups were larger than in pure berberine groups; and there is a similar result existed in the sham-operated group. It stated that the compatibility of HLJDT has contributed to the diverse pharmacokinetics

characteristic of berberine, and generated synergies. In view of drug interaction *in-vivo*, it is necessary to pay more attention to drug compatibility on the absorption of medicine.

The affection of cerebrovascular disease on the pharmacokinetics of berberine was analyzed by the comparison of MCAO rats and sham-operated rats. The authors found that the absorption of berberine in MCAO group was better than in sham-operated group. Comparative pharmacokinetics parameters of berberine in MCAO rats and sham-operated rats after oral administration of HLJDT extract were as follows: AUC(0-t): (8.599 \pm 3.390)VS(6.581 \pm 0.841) $\mu\text{g/ml}\cdot\text{h}$; AUC(0- ∞): (14.433 \pm 9.347)VS(9.980 \pm 2.130) $\mu\text{g/ml}\cdot\text{h}$; C_{max} : (1.537 \pm 0.877)VS(0.927 \pm 0.338) $\mu\text{g/ml}$. One could find

Table 4. Pharmacokinetics parameter of berberine after oral administration of HLJDT decoction or pure berberine (258 mg berberine/kg body weight).

Parameter	Unit	MCAO group		Sham-operated group	
		Pure berberine	HLJDT extract	Pure berberine	HLJDT extract
AUC(0-t)	µg/ml*h	7.355±2.577	8.599±3.390	4.460±1.271	6.581±0.841
AUC(0-∞)	µg/ml*h	12.324±6.729	14.433±9.347	7.537±3.942	9.980±2.130
MRT(0-t)	h	9.678±0.785	9.715±1.991	9.422±1.255	9.529±2.330
MRT(0-∞)	h	22.231±3.932	21.993±9.881	19.047±6.629	19.830±7.558
t _{1/2Z}	h	14.177±3.484	16.086±7.047	12.517±4.535	14.047±7.578
Tmax	h	0.708±0.188	0.708±0.188	0.583±0.303	0.542±0.292
Cmax	µg/ml	1.022±0.560	1.537±0.877	0.742±0.403	0.990±0.452

a similar case after oral administration of pure berberine (Table 4). It is noticed that when the C_{max}, AUC of berberine in MCAO group increased significantly, the bioavailability of berberine in the body was greatly increased. To analyze the reason of different pharmacokinetics among MCAO rats and sham-operated rats, it may be related with cerebral ischemia. It is helps to achieve the active concentration which could express pharmacological effects. It proved that HLJDT and berberine could have therapy effect on cerebrovascular disease. Some similar pharmacokinetics characteristics have occurred in some other natural medicines and other diseases. Yu et al. (2008) had proved that diabetes was responsible for the enhancements in the bioavailability of the alkaloids of *R. coptidis* extract in rats, perhaps due to the pathological change in pharmacokinetics. It is prompted that people should consider the change of pharmacokinetics in different physical condition in clinical application.

The three extraction methods as follows were commonly used in pharmacokinetics study: Liquid-liquid extraction (LLE), solid phase extraction (SPE), and protein precipitation (PPT). In the present study, the author compared the aforementioned methods and the extraction organic solvents such as ethyl ether-sodium hydroxide solution (Lu et al., 2006), acetonitrile (Chen et al., 1995; Gupta et al., 2009), ethyl ether (Hua et al., 2007), solid phase extract by methanol and acetonitrile-methanol mixture (Deng et al., 2008). The results showed that adopting methanol to precipitate protein, the composition had a great retain, and the recovery of berberine was acceptable. On the other hand, ample handling was simple and could minimize the error in the extraction. Therefore the authors adopted the LLE by methanol in the study.

The MCAO rat model chose in this paper is believed to be one of the most reliable and commonly used models in the study of cerebral ischemia (Wu and Jia, 2007). In addition, the effect of MCAO was influenced by the kinds of animals. Markgraf et al. (1993) study found that Wistar

rat's MCAO has smallest average of cerebral infarction area and largest variability. Fisher-334 rat has largest MCAO average of cerebral infarction area and smallest variability. SD rat is between the two. In respect to the quality of animal, nowadays most of researchers regard the 280 to 350 g of rats as idealities.

Acknowledging earlier published experience with berberine in Xu et al. (2000) and Wei et al. (1995) researches, where the author found that HLJDT extract 8 g/kg (is equal to 32.65 g herbs/kg) had better protective effect against impairment of learning and memory induced by transient cerebral ischemia in mice and ip of 100 mg berberine/kg body weight in four doses showed a good protective effect on rat hippocampus against cerebral ischemia. So the author decided that oral administration was at the dosage of 258 mg berberine/kg body weight.

To sum up, results of the present study indicated disease and other herbs in HLJDT would influenced the potential pharmacokinetics of berberine. The prescription of HLJDT increased the bioavailability of berberine *in vivo*, and the influence of cerebrovascular disease helped the incremental absorption of berberine which would improve the therapeutic efficacy and directly compromise drug usage in clinics.

Conclusion

In conclusion, significant differences were found in this study between the model and sham-operated group. The author had developed a rapid, sensitive method to assay the concentration of berberine in rat plasma. It was the first time to analyze the influence of cerebrovascular disease on the pharmacokinetics of berberine. In the meanwhile, the author has evaluated the drug interaction in HLJDT. This study may give an advice for future herbal research which will be carried on the same subject. And related research findings would be reported in another treatise. It made the foundation of the safety of herbal

application in clinic.

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