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Distribution of liposomal breviscapine in brain following intravenous injection in rats

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Abstract

Aim: To investigate distribution of breviscapine in brain after intravenous (i.v.) injection of liposomes.
Methods: Breviscapine liposomes were prepared by rotary evaporation–sonication method. Particle size, encapsulation efficiency and stability of liposomes were respectively examined. In vitro drug release was investigated in 0.9% sodium chloride at 37 °C. Rats were divided into two groups. Liposomes were given to one group and commercial injection (*Injectio Breviscapine*) was given to the other at a single dose of 28.1 mg kg^{-1} i.v., respectively. Scutellarin in rat brain at different sampling time was determined by RP-HPLC. The brain concentration–time curves of breviscapine liposomes and commercial injection were constructed and pharmacokinetic parameters were calculated and compared by statistic analysis.
Results: The average liposome diameter was 735 ± 59 nm and encapsulation efficiency was 85.1 ± 2.3%. The average accumulative release percentage of breviscapine liposomes in 0.9% sodium chloride was less than 30% within 24 h. The mean concentration–time curves of breviscapine liposomes and commercial injection were both fitted to one-compartment model. There are significant difference of parameter $T_{1/2}$ and AUC_{0-360} between liposome and commercial injection ($p < 0.05$). $T_{1/2}$ of breviscapine liposomes and commercial injection were 23.13 ± 7.71 and 6.27 ± 1.84 min, respectively. The brain AUC ratio of breviscapine liposomes to commercial injection was 443.4 ± 92.3%.
Conclusion: Compared with the commercial injection, liposomes delivered more drugs into the brain and have longer elimination time.

Keywords: Breviscapine; Scutellarin; Liposomes; Pharmacokinetics; RP-HPLC

1. Introduction

Breviscapine is the flavonoid constituents extracted from Chinese herb *Erigerin breviscapus* (Vant.) Hand-Mazz. It contains mainly scutellarin and a small quantity of apigenin-7-*O*-glueuronide. Scutellarin is the primary active ingredient of breviscapine (Zhang et

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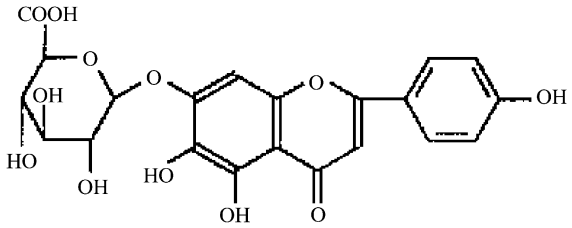

Fig. 1. Structure of scutellarin.

al., 2000). The structure of scutellarin was shown in Fig. 1. It is a sort of flavone glycoside. In China, the preparations of breviscapine (*Injectio Breviscapine* and *Breviscapine Tablets*) are extensively used for the treatment of cerebrovascular diseases such as paralysis caused by cerebral infarction, hypertension and chronic arachnoiditis along with their sequelae (Zhang et al., 2002). It has been reported that breviscapine possesses anticoagulation effect (Zhou et al., 1992) and can protect against cerebral ischemia-reperfusion injury by many pathways of action (Shi et al., 1998; Shuai and Dong, 1998). In recent years, many studies have provided evidences for the neuroprotective effects of scutellarin. It has been reported scutellarin exerts a potent protective effect against oxidative damage in synaptosomes induced by superoxide (Liu et al., 2003a). The neuroprotective properties of scutellarin have been explored at the cellular level (Hao and Liu, 2004).

However, it has been reported that the absolute bioavailability of breviscapine oral preparations was only $0.4 \pm 0.19\%$ (Ge et al., 2003). The pharmacokinetics of breviscapine at a single intravenous dose to rabbits and dogs have also been studied (Li et al., 2003; Liu et al., 2003b). It was shown that scutellarin eliminated rapidly in the blood and the plasma concentration–time curve was fitted to a three-compartment model, which indicated the fast distribution of scutellarin in vivo. The result of biodistribution study of breviscapine in mice after intravenous injection showed that most of ^{3}H-scutellarin accumulated in the cholecyst, intestine and dejecta within 1 h, and 41.2% of ^{3}H-scutellarin was found in the excrement and urine within 24 h (Cai, 1981).

Liposomes have long been used as drug delivery system (DDS) to realize long-circulation and target-delivery of drugs (Aquilur et al., 1986). In order to prolong the residence of breviscapine in blood and deliver more breviscapine into brain, breviscapine liposomes were prepared and the pharmacokinetic behavior of breviscapine liposomes and commercial injection (*I. Breviscapine*) in rat brain were compared.

2. Experimental

2.1. Materials

Breviscapine was purchased from Yunnan Plant Pharmaceutical Industry Ltd. (Yunnan, China). Soybean phosphatidylcholine was purchased from Shanghai Taiwei Pharmaceutial Industry Ltd. (Shanghai, China, PC > 92%). Cholesterol was obtained from Shanghai Chemical Reagent Co. (Shanghai, China). *I. Breviscapine*, an injection solution of scutellarin (20 mg/5 mL), was manufactured by Dilong Pharmacy Ltd. (Heilongjiang, China). Scutellarin (purity 96.4%) and rutin (internal standard, IS) were purchased from National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and acetonitrile were of HPLC-grade. NaH_2PO_4 and other chemicals used were of analytical grade. Water was double distilled.

2.2. Animals

Wistar rats (200 ± 20 g body weight) were provided by the Animal Center of China Pharmaceutical University).

2.3. Chromatographic conditions

The concentration of scutellarin was determined by HPLC. The HPLC system was consisted of two pumps (HP1100, Agilent, USA), a Diamonsil C18 column (4.6 mm × 250 mm, 5 μm, Dikma, Beijing, China) with a Shim-park C18 precolumn (4 mm × 3.0 mm, 5 μm, Simadzu, Tokyo, Japan) maintained at 35 °C, an UV detector (HP1100, Agilent, USA) at 335 nm and an autosampler (HP1100, Agilent, USA). The HP1100 ChemStation software was applied on the HPLC system. The mobile phase consisted of methanol–acetonitrile–0.5%phosphoric acid (35:10:65 v/v/v) was delivered at a flow rate of 1.0 mL min^{-1}.

2.4. Preparation of breviscapine liposomes

Breviscapine liposomes were prepared by a rotary evaporation–sonication method. In brief, appropriate amount of lecithin (187.5 mg), cholesterol (93.8 mg) and breviscapine (37.5 mg) were dissolved in dichloromethane. Then the mixture was dried to be a thin film in the rotary evaporation apparatus under vacuum in water bath at 37 °C. The resulting film was hydrated with 0.9% NaCl solution at 37 °C for 2 h to make a coarse lipid suspension. After the extrusion was carried out at 4 °C in the homogenizer (AVESTIN Co., Canada), the final unilamellar liposomal vesicles were filled into cillin bottles (1 mL for each) together with cryoprotectant of Mannitol and were freeze-dried in the freeze dryer (Labcomb Instrument Ltd., USA).

2.5. Physicochemical properties of breviscapine liposomes

The shape of breviscapine liposomes was observed by the transmission electron microscope (Submicron particle sizer–Nicomp particle sizing system, USA). The particle size of breviscapine liposomes was measured by dynamic light scattering (PCS, Malven Instrument Ltd. BK).

The encapsulation efficiency of breviscapine liposomes after reconstitution was determined by dialysis. A 0.5 mL of breviscapine liposomes suspension was added into a dialytic membrane bag with 0.9% NaCl as dialysis medium. The dialysis was carried out at 37 °C for 4 h. The unencapsulation efficiency of breviscapine liposomes was calculated as the ratio of the amount of free scutellarin in dialyzing medium to total scutellarin in 0.5 mL liposome suspension. Then the encapsulation efficiency was calculated according to the following equation:

$$\mathrm{EE}\% = \left(1 - \frac{M_{\mathrm{free}}}{M_{\mathrm{total}}}\right) \times 100\%$$

where M_{total} is the total amount of scutellarin in breviscapine liposomes and M_{free} is the amount of free scutellarin not encapsulated in the liposomes. The amount of scutellarin in liposomes was determined by HPLC with dissolving liposomes in methanol and the amount of free scutellarin in dialyzing medium was also determined by HPLC.

The in vitro release of breviscapine liposomes was analyzed according to the published method (Zhang et al., 2001). In short, 0.5 mL of breviscapine liposomes suspension was placed in a dialysis membrane bag with 0.9% NaCl as dissolution medium. The temperature of dissolution medium was maintained at 37 °C with the dialysis bag stirring for 24 h. At scheduled intervals, 1 mL of the dissolution medium was collected and the same volume of dissolution medium was added immediately. The concentrations of scutellarin in dissolution medium were analyzed by HPLC. The release percentage was calculated according to the following equation:

$$\text{Drug release } (\%) = \left(\frac{M_{\mathrm{release}}}{M_{\mathrm{total}}}\right) \times 100\%$$

where M_{total} is the total amount of scutellarin in breviscapine liposomes and M_{release} is the amount of scutellarin released from breviscapine liposomes into dialysis medium at any time.

2.6. Drug administration and biological samples collection

The animal experiments had gained the China ethic committee's approval. Twelve rats (200 ± 20 g) were randomly divided into two groups to be injected with breviscapine liposomes and commercial injection (*I. Breviscapine*, a sort of breviscapine solution), respectively. The rats were anaesthetized with ethyl carbamate (15% w/w, 1 mL 100 mg^{-1} body weight) and securely positioned on a surgical table. Midline incisions in the neck and gaskin skin were performed, and then the carotid artery and femoral veins were separated. Before administration one carotid artery was ligated at the end near the brain and a polyethylene cannula was placed in it toward the heart for the blood sampling. Breviscapine liposomes or commercial injections were respectively injected into the femoral vein at a single dose of 28.1 mg per 1.0 kg body weight and blood samples were taken from the carotid arteries at 2, 5, 10, 15, 30, 60, 90, 120, 180 and 240 min following injection.

One hundred and thirty-two rats (200 ± 20 g) were divided into 12 teams averagely. Six teams named Lips were administered breviscapine liposomes and the other six teams named Com were administered commercial injection via femoral vein. In the first week

(period I), experiments were carried out on three Lips teams and three Com teams. And the remnant teams were examined in the second week (period II). The rats were anaesthetized with ethyl carbamate and securely positioned on a surgical table. Midline incisions in the gaskin skin were performed, and then the femoral veins were separated. Breviscapine liposomes or commercial injection (a sort of breviscapine solution) were respectively injected into the femoral vein of the rats at a single dosage of 28.1 mg per 1.0 kg body weight. After administration, the rats were sacrificed by cervical dislocation at the time intervals of 2, 5, 7, 10, 15, 20, 30, 45, 60, 120 and 360 min, respectively, and the cerebra were collected and weighted. At each sampling time, six rats were sacrificed and sampled ($n = 6$).

2.7. Sample pretreatment

Rutin has the analogous structure with scutellarin and was used as internal standard. A 30 μL of rutin methanol solution (15 μg mL^{-1}) and 20 μL of 5 M phosphoric acid (Dafang et al., 2003) was added to each brain or blood sample (800 μL). Then 1.0 mL methanol was added to precipitate proteins. After the samples were vortex mixed for 1 min, the mixture was centrifuged for 10 min at 10,000 rpm. A 0.8 mL of the upper phase was transferred to another tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was dissolved in 120 μL mobile phase and vortex mixed. A 20 μL of the solution was injected into the HPLC system for analysis.

2.8. Validation of analytical method

Stock solution of scutellarin (250 μg mL^{-1}) was prepared by dissolving 12.5 mg of scutellarin in a 50 mL volumetric flask and then was diluted with methanol to obtain 0.2, 1.0, 2.0, 4.0, 10.0, 20.0 and 30.0 μg mL^{-1} of standard scutellarin solution. The standard samples at different concentrations of 5, 25, 50, 100, 250, 500 and 750 ng mL^{-1} were prepared by adding 20 μL of the corresponding standard scutellarin solution into 0.8 mL of blank brain plasma. After pretreatment as formally described, the calibration curves were constructed using a $1/y^2$ weighted linear regression of the peak–area ratios versus the brain plasma concentration of the analyte.

The relative and absolute recoveries, within-day and between-day precisions were all calculated by analyzing the quality control samples (QC samples) at concentrations of 5, 250 and 750 ng mL^{-1}. Each control level was prepared with number of 5. At the same concentrations, the freeze-thaw stability was evaluated after undergoing three freeze (−20 °C) – thaw (ambient) cycles.

2.9. Data processing and statistical analysis

The brain concentration–time data of breviscapine liposomes and commercial injection were fitted by 3P97 pharmacokinetics program (the Section of Mathematical Pharmacology of Chinese Mathematical Pharmacological Society), and the pharmacokinetic parameters were calculated. The area under the concentration–time curve ($AUC_{0–360}$) was determined with trapezium method. The one-way analysis of variance (ANOVA) was conducted to compare the major pharmacokinetic parameters.

3. Results and discussion

3.1. Factors on the encapsulation efficiency of breviscapine liposomes

Cholesterol is a necessary component of liposomes, which makes the liposome membrane tight and compact so that the drug encapsulated will not leak out. Breviscapine liposomes with various mol ratios of PC to CHOL were prepared and their encapsulation efficiencies were determined. Results showed that the average encapsulation efficiency was the highest 85.1 ± 2.3% when the mol ratios of PC to CHOL was 1:1.

The drug/lipid weight ratios can also affect encapsulation efficiency of liposome. The average encapsulation efficiency was the maximum 85.1 ± 2.3% when the drug/lipid weight ratio was 1:5.

3.2. Shape and size of breviscapine liposomes

The shape of breviscapine liposomes was round or oval observed by the transmission electron microscope (20,000×) as shown in Fig. 2. The average vesicle diameter was 735 ± 59 nm determined by

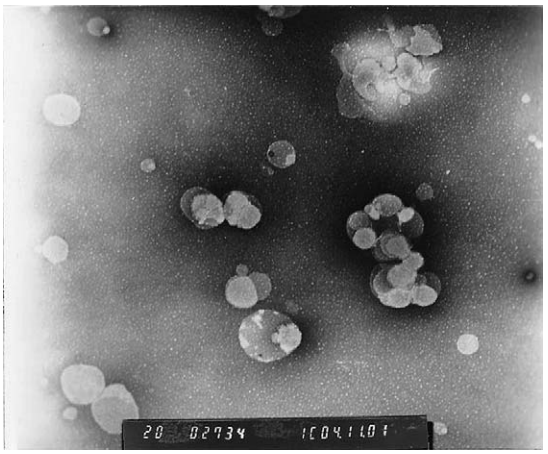

Fig. 2. Transmission electron microscope photograph (20,000×) of breviscapine liposomes.

dynamic light scattering and the polydispersity index was 0.208 ± 0.083.

3.3. In vitro release of breviscapine liposomes

The result of in vitro release was shown in Fig. 3. The scutellarin released slowly from breviscapine liposomes and the accumulative release percentages were less than 30% within 24 h. On the other hand, the accumulative release percentage of free breviscapine was 100% within 4 h. This demonstrated that scutellarin could pass through the dialysis membrane entirely into the dissolution medium in time and scutellarin can be released from liposomes slowly.

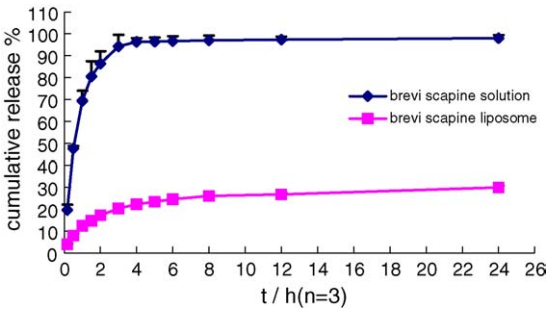

Fig. 3. Results of cumulative release of breviscapine liposome ($n = 3$).

3.4. Sample pretreatment

The sample pretreatment prior to the HPLC analysis is vital to the determination of biological samples, especially the tissue. We used methanol to denature and precipitate proteins of rat brain or blood. Phosphoric acid (5 M, 20 μl) was added before solvent mixtures, not only to adjust the pH of brain samples, but also to denature and precipitate proteins, which might help the bound scutellarin to dissolve in the organic solvent.

3.5. Validation of analytical method

3.5.1. Assay specificity

The assay specificity was demonstrated by comparing the HPLC chromatograms of three samples given in Fig. 4. It was shown that there were no interferences with scutellarin and internal standard. The retention time for scutellarin and internal standard were 11.5 ± 0.5 and 20.5 ± 0.5 min, respectively.

3.5.2. Linearity and sensitivity

The calibration curves of scutellarin in brain of rats had good linearity over the range of 5–750 ng mL^{-1}. The regression equation was $y = 0.004008x + 0.001721$ ($r = 0.9949$, $n = 5$). The lowest limit of quantitation (LLOQ) was 5 ng mL^{-1}, defined as the peak–area ratios of scutellarin versus noise was 10:1.

3.5.3. Recovery and precision

For the low, medium and high concentration, the recoveries were 81.71 ± 7.30, 104.09 ± 5.64 and 102.52 ± 2.94%. The extraction recoveries were 87.35 ± 14.24, 87.47 ± 2.04 and 96.31 ± 3.00%. The within-day RSD was less than 10.0%, and that of the between-day was less than 15.93%.

3.6. Blood pharmacokinetics

The plasma concentration–time profile of breviscapine liposomes was shown in Fig. 5 and commercial injection was used as a control. Both curves fit the open two-compartment model. The major pharmacokinetic parameters were listed in Table 1. The plasma AUC ratio of breviscapine liposomes to commercial injection was 443.4 ± 92.3%. The pharmacokinetic parameters of commercial injection showed that breviscapine was

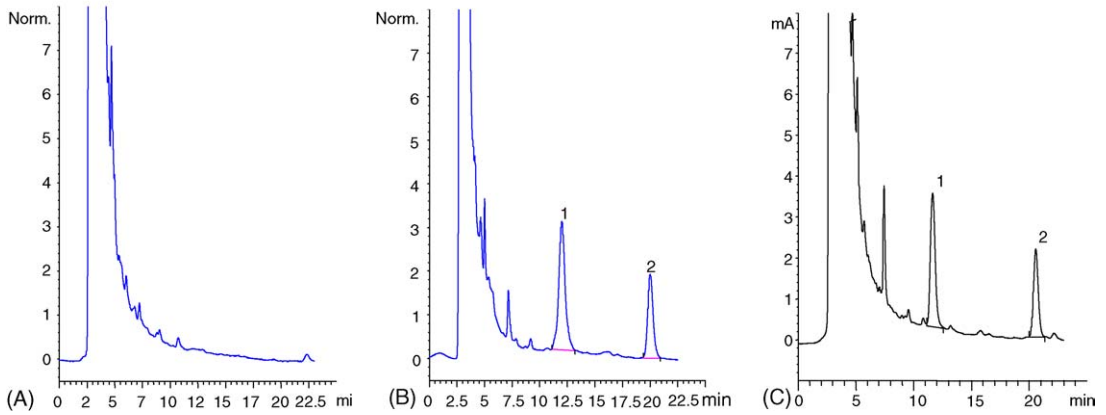

Fig. 4. HPLC chromatograms of blank brain plasma (A), blank brain plasma with 500 ng mL^{-1} scutellarin and internal standard were added (B) and a brain sample after i.v. 2 min to a rat (C). Peaks 1: scutellarin; 2: IS.

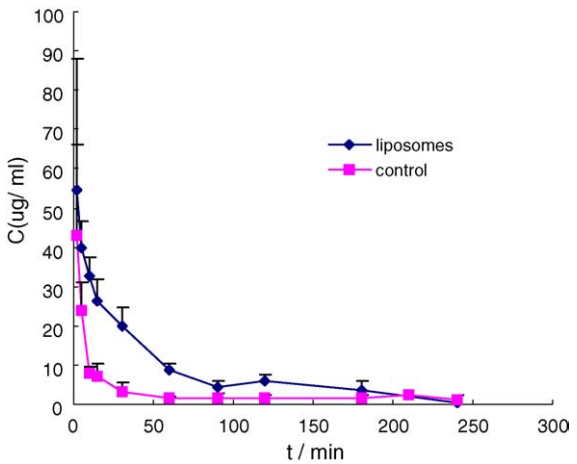

Fig. 5. Mean blood concentration profile in rats after intravenous injection of breviscapine liposomes and commercial injection ($n = 6$).

rapidly cleared in bloodstream. Such results were corresponding to those of ^{3}H-scutellarin analysis in which the large amount of radioactivities was recovered in cholecyst and intestine after i.v. 1 h. On the other hand, the pharmacokinetic parameters of breviscapine liposomes showed that the liposome did increase the breviscapine concentrations in plasma, retarded the clearance and exhibited the properties of sustained-release.

Table 1
Blood pharmacokinetic parameters of scutellarin after i.v. breviscapine liposomes and commercial injection in rats ($n = 6$)

Parameters	Breviscapine liposomes	Commercial injection
$V_{(c)}$ (mL)	25.47 ± 2.1	28.2 ± 3.1
AUC (μg min mL^{-1})	1946 ± 208	633 ± 35
$CL_{(s)}$ (mL min^{-1})	0.995 ± 0.13	1.76 ± 0.16

3.7. Cerebric pharmacokinetics

The mean brain concentration–time curves of breviscapine liposomes and commercial injection were protracted as shown in Fig. 6. The brain concentration of commercial injection decreased quickly to 20 ng mL^{-1} within 30 min, and after that, it went down very slowly. As to the breviscapine liposomes, the brain concentration decreased more slowly than that of commercial injection. Even at 2 h after administration, the brain concentration was still as high as that of commercial injection after 15 min of administration.

The mean concentration–time curves of breviscapine liposomes and commercial injection were

Table 2
Cerebric pharmacokinetic parameters of scutellarin after i.v. breviscapine liposomes and commercial injection in rats ($n = 6$)

Parameters	Breviscapine liposomes	Commercial injection
$T_{1/2}$ (min)	23.12 ± 7.71	6.27 ± 1.84
K_e (min^{-1})	0.03379 ± 0.0142	0.1186 ± 0.0348
C_0 (ng mL^{-1})	510.32 ± 92.21	322.19 ± 105.67
AUC_{0-36} (ng min)	22755.43 ± 5501.8	5131.98 ± 781.23

Table 3
Results of ANOVA of cerebric pharmacokinetic parameters

Variance	d.f.	F			$a = 0.05$
		AUC_{0-360}	$T_{1/2}$	C_0	
From subjects	5	0.530217	0.555554	5.154573	$F_{0.05}(5,4) = 6.26$
From periods	1	0.310596	0.047449	9.904493	$F_{0.05}(1,4) = 7.71$
From preparations	1	116.0885	29.51051	37.91425	

both fitted to one-compartment model with the main pharmacokinetic parameters as follows: $T_{1/2}$ were 23.13 ± 7.71 and 6.27 ± 1.84 min, respectively; AUC_{0-360} were 22755.43 ± 5501.8 and 5131.98 ± 781.23 ng min mL^{-1}, respectively, which were listed in Table 2.

The result of statistical analysis of main pharmacokinetic parameters was shown in Table 3. It was shown that AUC_{0-360} and $T_{1/2}$ were similar and had no significant difference ($a = 0.05$) between rats and periods. C_0 were similar too and had no significant difference between rats, but differed significantly between periods. There were significant differences of AUC_{0-360}, $T_{1/2}$ and C_0 ($a = 0.05$) between preparations. AUC_{0-360} and C_0 of the breviscapine were significantly improved and the $T_{1/2}$ was elongated by liposomes.

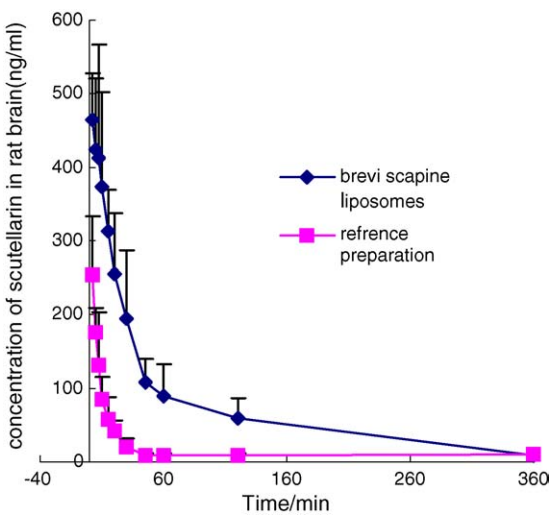

Fig. 6. Mean brain concentration–time curve of scutellarin after i.v. breviscapine liposomes and commercial injection in rats ($n = 6$).

4. Conclusion

The breviscapine liposomes had high encapsulation efficiency, small size and slow release property. In this study, a specific and sensitive assay using RP-HPLC for the determination of scutellarin in rat brain was developed. Compared with the reference injection, liposomes delivered much more scutellarin into brain and retained fairly high concentration for certain length of time. It was shown that breviscapine liposomes were a promising drug carrier of the drug targeting and sustained-release properties.

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