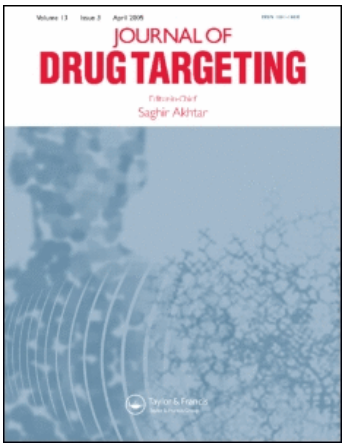

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Preparation, characterization, and biodistribution of breviscapine proliposomes in heart

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RESEARCH ARTICLE

Preparation, characterization, and biodistribution of breviscapine proliposomes in heart

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Abstract

Breviscapine proliposomes were prepared by ethanol injection–homogenization–lyophilization method. On contact with 5% glucose, the proliposomes were rapidly converted into a liposomal dispersion, in which a certain amount of breviscapine was entrapped by the liposomes. The entrapment efficiency measured by reverse dialysis method was 77.89 ± 0.28%. The particle size, polydispersity index, and zeta potential of breviscapine liposomes were 504.83 ± 52.88 nm (by intensity), 0.17 ± 0.02, and −(20.31 ± 1.03) mV, respectively (mean ± SD, $n = 3$). In mimic-biomembrane model experiment, breviscapine was distributed not only to n-octanol and buffer phase but also to interfacial phase. After bolus administration, the elimination phase ($t_{1/2(\beta)} = 66.386$) of liposomal formulation in plasma was 4.8 times longer than that of solution formulation ($t_{1/2(\beta)} = 13.695$). The AUC and MRT values of liposomal formulation in heart were increased more than 11.7- and 3.2-fold versus solution formulation, respectively. These results were all beneficial to heart disease therapy.

Keywords: *Breviscapine; proliposomes; reverse dialysis; mimic-biomembrane; biodistribution in heart*

Introduction

Breviscapine, a flavone glucuronide, extracted from a Chinese herb *Erigeron breviscapus* (Vant.) Hand-Mazz. (Zhang et al., 1988), is widely used in the treatment of angina pectoris, coronary heart disease, and cerebral infarction and its sequelae (Zhang, Li, & Zhang, 2002). Breviscapine and its commercial injection (*Injectio Breviscapine*) were listed in the Pharmacon Criteria (Chinese Traditional Patent Medicine). It contains mainly scutellarin (primary active ingredient) and little apigenin-7-*O*-glucuronide. The structure of scutellarin (4′,5,6-tetrahydroxyflavone-7-*O*-glucuronide) is shown in Figure 1. Breviscapine shows wide pharmacological effects. It significantly reduces ST-segment elevation and infarction size in hearts subjected to myocardial infarction caused by left coronary artery ligation, significantly decreases oxygen consumption in myocardium, and significantly reduces lactate dehydrogenase leakage, intracellular free Ca^{2+} levels, and apoptosis and necrosis in cardiomyocytes subjected to hypoxia (Li et al., 2004).

The result of biodistribution study of scutellarin in mice after intravenous injection showed that a majority of ^{3}H-scutellarin were accumulated in the cholecyst, intestine, and dejecta, whereas only a little of it was found in the heart (Cai, 1981). The transformation of a free drug into its liposomal formulation is a strategy to control and modify its pharmacokinetics and tissue distribution. The major therapeutic research on liposomes was focused on tumor, lymphatic, liver, and brain targeting and gene delivery in recent years (Haynes et al., 2008; Kaur, Nahar, & Jain, 2008; Zhao et al., 2008; Ko, Bhattacharya, & Bickel, 2009; Zheng et al., 2009). Liposomes also have advantages for heart disease therapy. Some animal experiments (Palmer et al.,

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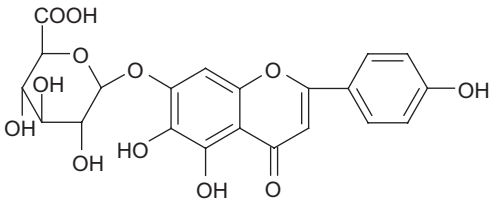

Figure 1. Structure of scutellarin.

1981; Cole et al., 1982; Caride, Twickler, & Zaret, 1984) have already demonstrated that the accumulation of liposomes in ischemic tissues is rather a general phenomenon and might be explained by impaired filtration in ischemic areas, which results in trapping of liposomes within those areas (Palmer et al., 1984). This observation led to the conclusion that drug-loaded liposomes could be used for "passive" drug delivery into the ischemic tissues, primarily into the infarcted myocardium (Palmer, Caldecourt, & Kingaby, 1984; Baldeschweiler, 1990). Liposomal formulations are helpful to trap breviscapine within infarcted myocardium tissue, resulting in an increase in therapeutic efficacy.

In this study, based on the conventional characteristics of breviscapine liposomes, the distribution of drug in each phase of mimic-biomembrane model *in vitro* and pharmacokinetics and biodistribution of drug in heart *in vivo* were investigated and compared with breviscapine solution (*Injectio Breviscapine*).

Materials and methods

Materials

Breviscapine was provided by Jiangsu Chia-tai Tianqing Pharmaceutical Co. Ltd. (Jiangsu, China). Scutellarin standard (purity > 98%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Lipoid E80 (egg yolk lecithin with 80–85% of phosphatidylcholine) was purchased from Lipoid GmbH (D-Ludwigshafen, Germany). Cholesterol and Tween 80 were obtained from Shanghai Chemical Reagent Co. (Shanghai, China). Dialysis bag (8000–10,000 MW cutoff, 25 mm) was purchased from Sigma. *Injectio Breviscapine*, which is an injection solution of scutellarin (20 mg/5 mL), was obtained from Gejiu Bio-Medicine Industry Ltd. (Yunnan, China). Other chemicals used were of analytical grade.

Preparation of proliposomes

An aliquot of 5 mL ethanolic solution of 20 mg breviscapine, 200 mg Lipoid E80, 60 mg cholesterol, and 100 mg Tween 80 was injected into well-stirred 50 mL of 0.8% cryoprotective agent mannitol and 0.04% EDTA aqueous solution in a thermostated bath at 37°C. After the evaporation of ethanol, the mixture was passed through a homogenizer (APV-2000; APV, Copenhagen, Denmark) with a first stage pressure of 1200 bar and a second stage pressure of 200 bar for five times. Finally, the homogenized mixture was filled into cillin bottles (10 mL per bottle) and fast frozen at –20°C for 24 h, and then the samples were moved to the freeze-drier (Heto FD2.5; Heto High Technology of Scandinavia, Birkerod, Denmark). The drying time was controlled 24h and the breviscapine proliposomes were obtained. Breviscapine liposomes (0.2 mg/mL scutellarin) obtained from rehydrated proliposomes with 5% glucose were used for the next experiment.

Chromatographic system

The chromatographic system (Xiong et al., 2006) consisted of a Waters 510 HPLC pump and a Waters 486 Absorbance UV detector (Waters Corp., Milford, MA). The wavelength of this detector was set to 335 nm. The high-performance liquid chromatography (HPLC) system was controlled using the Millennium 2010 ChemStation software. The analytical column was a reverse phase Hypersil C_{18} column (250 × 4.6 mm, 5-μm particle size; Dalian Elite Analytical Instrument Co., Ltd., Dalian, China) maintained in a column oven (Timberline Instruments, Boulder, CO) and protected by a guard column (10 × 4.6 mm) packed with the same material. The mobile phase was composed of methanol/water/glacial acetic acid (40:60:1). Elution was performed isocratically at 40°C at a flow rate of 1.0 mL/min.

Size distribution and zeta potential of liposomes

The mean particle size, polydispersity index, and apparent surface potentials of the liposomes were estimated by using a Malvern Zetasizer 3000 (Malvern Instruments Ltd., Worcestershire, UK) at 25°C after resolving the proliposomes with 5% glucose.

Entrapment efficiency

The entrapment efficiency (EE %) of scutellarin in the liposomes was determined by reverse dialysis method (Xiong et al., 2004) with minor modifications. For determining the equilibration time of reverse dialysis, a dialysis bag containing 2 mL of 5% glucose (inner phase) was kept in 50 mL of liposomes (outer phase) and stirred continuously. An aliquot of 25 μL of each concentration of scutellarin solution was withdrawn from the inner phase at appropriate intervals, immediately replaced with 25 μL fresh 5% glucose and measured using HPLC. The time at which the concentration of scutellarin in inner phase did not increase further was determined as the equilibration time. For determining the recovery of scutellarin sample in blank liposomes (prepared in the same manner as

described earlier, except that breviscapine was not added), three dialysis bags containing 2 mL of 5% glucose were kept in three 50 mL physical mixture of blank liposomes and different concentrations of scutellarin, respectively. At the equilibration time of dialysis, outer phase was stirred continuously, then 25 μL of breviscapine solution was withdrawn from the inner phase and the concentration of scutellarin in that solution was measured using HPLC. The recovery was calculated according to the following equation:

$$\text{Recovery } \% = [(C_{\text{inner}} \times V_{\text{total}})/(C_{\text{outer}} \times V_{\text{outer}})] \times 100\%,$$

where C_{inner} is the concentration of scutellarin in inner phase at equilibration time of dialysis; C_{outer} is the concentration of scutellarin in outer phase before dialysis; V_{total} is the total volume of dialysis system (52 mL); and V_{outer} is the volume of outer phase before dialysis (50 mL).

The procedures applied for determining the entrapment efficiency were similar to those procedures used for determining recovery except that breviscapine liposomes (50 mL) were added to the outer phase instead of the physical mixture. The concentration of scutellarin in liposomes before dialysis was also measured using HPLC by dissolving liposomes in 10% Triton X-100 in ethanol. Then, the entrapment efficiency was calculated according to the following equation:

$$\text{EE } \% = [(C_{\text{total}} \times V_{\text{outer}} - C_{\text{free}} \times V_{\text{total}})/ (C_{\text{total}} \times V_{\text{outer}})] \times 100\%,$$

where C_{total} is the concentration of scutellarin in liposomes before dialysis; C_{free} is the concentration of scutellarin in inner phase at equilibration time of dialysis; V_{outer} is the volume of outer phase before dialysis (50 mL); and V_{total} is the total volume of dialysis system (52 mL).

Scanning electron microscopy and transmission electron microscopy

For observing the surface morphology of proliposomes, the lyophilized cake was crushed and examined by scanning electron microscopy (SEM) after coating them with gold in a sputter coater at 20 kV with JFC-1100 (Jeol, Japan). Transmission electron microscopy (TEM) observation of liposomes was carried out at 75 kV with H-7000 (Hitachi, Japan), which was negatively stained with 2% phosphotungstic acid and placed on a copper grid coated with film.

Dynamic partition experiment in n-octanol/buffer system

n-Octanol was used to represent the biomembrane. n-Octanol and PBS (pH 7.4) were co-saturated with each other for 24 h at 37°C before use. n-Octanol (25 mL) was shaken with 25 mL buffer solution containing solution or liposomes of breviscapine (0.12 mg/mL scutellarin) at 37°C. At appropriate intervals, the same amount of n-octanol phase and buffer phase was removed, respectively, and the concentration of scutellarin in each phase was determined using HPLC. The amount of scutellarin at the interface between n-octanol and buffer phase was calculated according to the following equation (Yamamura, Nakao, & Yano, 1991):

$$M_{\text{i}} = M_{\text{t}} - M_{\text{o}} = M_{\text{w}}$$

where M_{i} is the amount of scutellarin at the interface; M_{t} is the amount of scutellarin added to the buffer phase; M_{o} is the amount of scutellarin in n-octanol phase; and M_{w} is the amount of scutellarin in buffer phase.

Pharmacokinetics and biodistribution of breviscapine liposomes in heart

To study pharmacokinetics and biodistribution of breviscapine liposomes in heart compared with breviscapine solution (*Injectio Breviscapine*), 12.5 mg/kg scutellarin in each preparation was injected as a single bolus via tail vein of Kunming mice. At appropriate intervals after administration, six animals were killed under ether anesthesia. Blood was collected in heparin-coated tubes and centrifuged at 1000 *g* for 5 min. Heart was removed, weighed, and homogenized (10%, w/v) in a solution of 1% sodium bisulfate in physiological saline. All samples were immediately frozen at –20°C until analysis. Blood and heart were processed and used for HPLC analysis (Xiong et al., 2006). In short, 10 μL of 10% sodium bisulfate solution was added to each 100 μL aliquots of mouse plasma sample to prevent oxidative degradation of scutellarin. A 100 μL aliquot of homogenate (10%, w/v) of heart, in a solution of 1% sodium bisulfate in physiological saline, was performed with a potter on ice. A 200 μL aliquot of methanol was added to precipitate the protein. Then, the samples were allowed to mix by shaking on a SW-80A vortex shaker (Shanghai Medical University Instrument Plant, Shanghai, China) for 5 min. After centrifugation at 10,000 *g* for 10 min at 4°C (Refrigerated Centrifuge 3K30; Sigma, German), a 20 μL aliquot of the supernatant fluid was injected into HPLC for assay. Pharmacokinetic parameters were determined using a log-linear trapezoidal method 3P97 (Mathematic Pharmacological Committee, Chinese Pharmacological Society, China).

Results and discussion

Preparation and characterization of proliposomes

The proliposomes in the present study were prepared by ethanol injection–homogenization–lyophilization

method. Ethanol injection method was more suitable for industrial procedures and more dispersible than the other methods (e.g., thin film hydration method). Tween 80 could improve the physical stability and decrease the particle size of liposomes. Breviscapine could be protected by EDTA against degradation *in vitro*. The lyophilized power was rapidly converted into a liposomal dispersion with mannitol as a cryoprotective agent. The entrapment efficiency, particle size, polydispersity index, and zeta potential of rehydrated liposomes were 77.89 ± 0.28%, 504.83 ± 52.88 nm (by intensity), 0.17 ± 0.02, and –(20.31 ± 1.03) mV, respectively (mean ± SD, $n = 3$).

Several methods have been used to determine the entrapment efficiency of liposomes, such as column chromatography (Sephadex G-50), conventional dialysis (liposomes in inner phase and dialysis medium in outer phase), and centrifugation. Generally, the outer phase of liposomes was diluted 20–100 times throughout the experiment to separate free drug and encapsulated drug by column chromatography or conventional dialysis. Significant dilution could lead to a leakage of breviscapine from liposomes. Although no dilution was performed during centrifugation, liposomes destroyed during centrifugation, which induces the reduction of entrapment efficiency, were measured. Therefore, the accuracy of entrapment efficiency determined by these three methods was in doubt. Throughout the whole reverse dialysis procedure, the dilution ratio of liposomes could be ignored (50 mL to 52 mL). The equilibration time of reverse dialysis was 6 h (Figure 2). The mean recovery of free drug in blank liposomes of three different concentrations was greater than 98% (Table 1). By using the method of Stewart (1980), we detected that there were no phospholipids in the inner phase at the equilibration time of dialysis, which demonstrated that liposomes could not pass through the dialysis membrane. These results demonstrated that the reverse dialysis method described was valid and suitable for studies on entrapment efficiency of breviscapine liposomes.

The rehydration of proliposomes progressed sufficiently rapidly and was evident at times as short as 30 sec after contact with 5% glucose. The amorphous lyophilized proliposome powders, as examined by SEM, are shown in Figure 3A. The porous structure of the cake allows rapid dispersion of the powder for aqueous reconstitution. The TEM of liposomes in 5% glucose is shown in Figure 3B. The particles showed a quasi-sphere. The TEM verified the presence of hollow lipid vesicles.

Table 1. The recovery of free drug in blank liposomes in reverse dialysis experiments.

C_{outer}[a] (mg/mL)	C_{inner}[b] (mg/mL)	Recovery[c] (%)
0.0197	0.0188 ± 0.0009	99.25 ± 4.84
0.0402	0.0380 ± 0.0019	98.22 ± 4.80
0.0598	0.0568 ± 0.0010	98.78 ± 1.66

[a]C_{outer} is the concentration of scutellarin in outer phase before dialysis.

[b]C_{inner} is the concentration of scutellarin in inner phase at the equilibration time of dialysis (mean ± SD, $n = 3$).

[c]Recovery % = $[(C_{inner} \times V_{total})/(C_{outer} \times V_{outer})] \times 100\%$ (mean ± SD, $n = 3$).

Dynamic partition experiment in n-octanol/buffer system

The ability of drugs to diffuse across membranes is frequently expressed in terms of their lipid–water partition

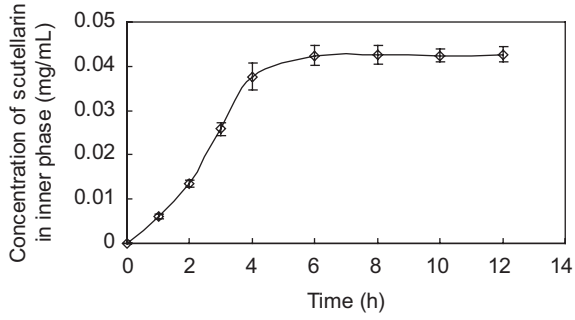

Figure 2. The concentration of scutellarin in inner phase of dialysis bag as a function of time in reverse dialysis experiments ($n = 3$).

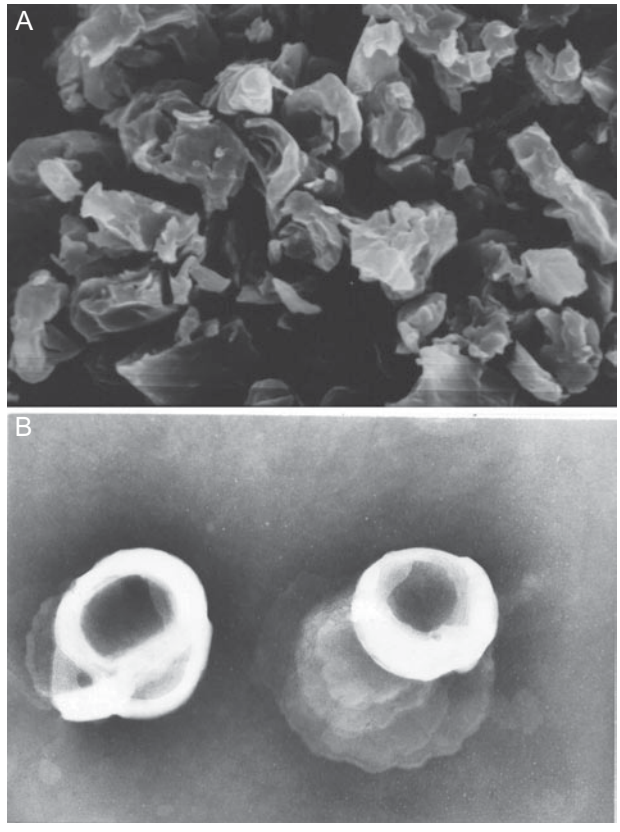

Figure 3. (A) SEM image of breviscapine proliposomes at ×500 magnification and (B) TEM image of liposomal breviscapine at ×20,000 magnification.

coefficient, which is a measure of the relative affinity of a drug for the lipid and aqueous phases. The n-octanol/buffer system is an adequate model for membrane-partitioning (Florence & Attwood, 1985; Barbato, La Rotonda, & Quaglia, 1997; Zheng, 1997; Lu, 1998; Thorsten & Johannes, 2004; Engelmann et al., 2007). In the present study, breviscapine was distributed not only to n-octanol and buffer phase but also to interfacial phase. After the partition equilibrium of breviscapine between three phases was established, the amount of breviscapine solution and liposomes in n-octanol phase was small (10.22 ± 1.17%, 11.67 ± 1.00% of total, mean ± SD, n = 3, respectively) and there was no significant difference between the two preparations ($P > 0.05$) (Figure 4A). This study demonstrated that there was no significant difference in the effectiveness to enter intracellular compartment for breviscapine between the two preparations. The amount of breviscapine of liposomes at interfacial phase was significantly higher than that of the solution ($P < 0.05$) after the partition equilibrium (Figure 4B). The amount of breviscapine liposomes was increased 1.29-fold to 38.12 ± 2.55% (mean ± SD, n = 3) of total at interface. The difference in the amount of breviscapine at interface between two preparations may contribute to the pharmacological effect difference on cell membranes.

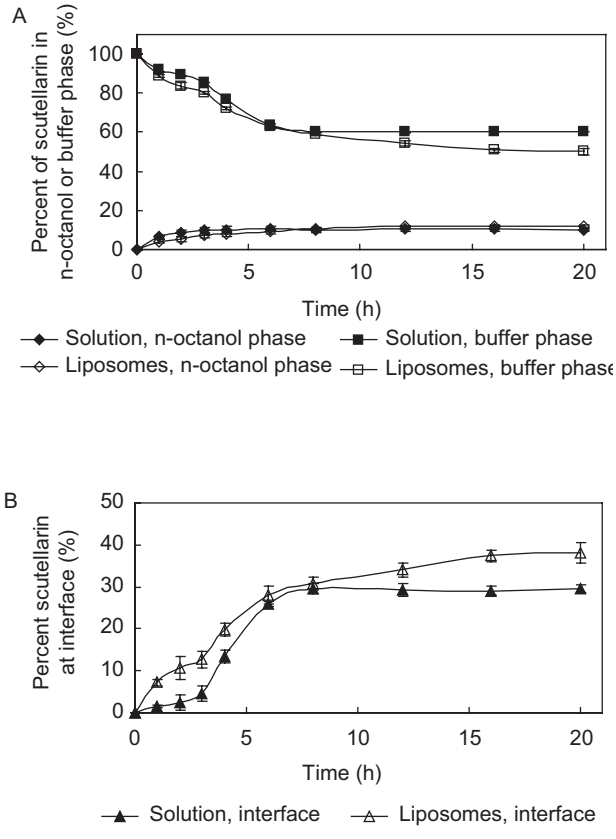

Figure 4. The amount of drug in n-octanol, buffer phase (A) and at interfacial phase between n-octanol and buffer phase (B) as a function of time in dynamic partition experiment (n = 3).

Breviscapine protect cardiomyocytes during myocardial injury through blocking calcium channel embedded in myocyte membrane (Wang et al., 2008). Liposomes may preferentially interact with cells via several mechanisms, including intermembrane transfer, adsorption, fusion, and endocytosis allowing drugs to internalize into cells and to localize into the plasma membrane (Pagano & Weinstein, 1978; Moreau & Cassagne, 1994). Hydrophilic drug entrapped in the aqueous space of liposomes can be sent to the cytoplasm, and the lipophilic drug in the lipid phase of liposomes may be sent to the plasma membrane (Tang et al., 1993). This experiment demonstrated that more breviscapines were transported to mimic-biomembrane (interface between n-octanol and buffer phase) by liposomal formulation. Whether more amount of drug in liposomes distribute to myocytes membrane than that in solution, higher chance of contacting with and effecting on channel embedded in cell membrane following more calcium channel inhibited calls for further investigation.

Pharmacokinetics and biodistribution of breviscapine liposomes in heart

Figure 5 and Table 2 report scutellarin concentration/time curves and targeting parameters for the two preparations. Breviscapine was very instable *in vivo*. Encapsulation of the breviscapine into liposomes

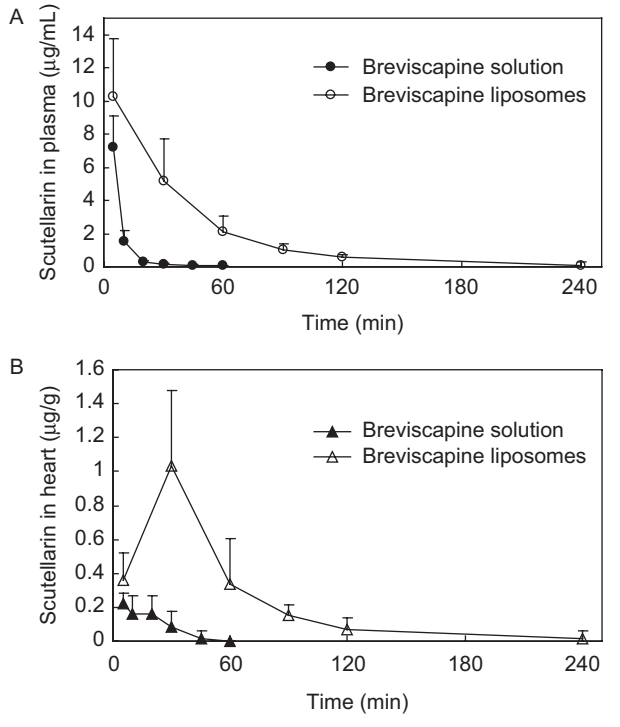

Figure 5. Pharmacokinetics (A) and biodistribution (B) of breviscapine solution and liposomes in heart after bolus administration (n = 6).

Table 2. Pharmacokinetic parameters and targeting parameters of breviscapine solution and breviscapine liposomes after bolus administration to mice (n=6).

Parameters	Breviscapine solution	Breviscapine liposomes
$V_{c\ plasma}$ (mL)	6.245	20.578
$t_{1/2(\alpha)\ plasma}$ (min)	1.954	12.192
$t_{1/2(\beta)\ plasma}$ (min)	13.695	66.386
$k_{21\ plasma}$ (1/min)	0.056	0.013
$k_{10\ plasma}$ (1/min)	0.322	0.027
$k_{12\ plasma}$ (1/min)	0.027	0.003
$Cl_{s\ plasma}$ (mL/min)	2.014	0.557
$AUC_{0\to T\ plasma}$ (μg·min/mL)	76.904	469.571
MRT_{plasma} (min)	6.059	38.918
$AUC_{0\to T\ heart}$ (μg·min/g)	5.130	60.237
MRT_{heart} (min)	17.137	55.596
TE[a]	0.067	0.128
TI[b]	11.742	

[a]TE (targeting efficiency) = AUC_{heart}/AUC_{plasma}.
[b]TI (targeting index) = $AUC_{heart\ (liposomes)}/AUC_{heart\ (solution)}$.

could protect it against degradation *in vivo* and therefore produced a significant change in pharmacokinetic parameters. After bolus administration, the liposomal formulation was more slowly removed from plasma compared with solution formulation. The elimination phase ($t_{1/2(\beta)}$ = 66.386) of liposomal formulation was 4.8 times longer than that of solution formulation ($t_{1/2(\beta)}$ = 13.695). The k_{10}, Cl_s, and mean residence time (MRT) values confirmed this trend. The biodistribution advantages in the heart of liposomes vehicle were obvious: the area under the concentration–time curve (AUC) and MRT values of liposomal formulation were higher than that of solution formulation. This conclusion was further confirmed by targeting parameters (Gallo et al., 1989; Gupta & Hung, 1989): targeting efficiency (TE, defined as the AUC in heart divided by the AUC in plasma) of liposomal formulation (TE = 0.128) increased more than 1.9-fold versus solution formulation (TE = 0.067) and the targeting index (TI, defined as the AUC of liposomes divided by the AUC of solution in heart) was 11.742. Increased amount and prolonged retention time of drugs in heart were all beneficial to heart disease therapy.

Conclusion

The ethanol injection–homogenization–lyophilization method has been proven to be appropriate for the encapsulation of breviscapine. The reverse dialysis method described is suitable for studies on entrapment efficiency of breviscapine liposomes. Breviscapine liposomes were distributed not only to n-octanol and buffer phase but also to interfacial phase in mimic-biomembrane experiment. On the basis of the results of pharmacokinetics and biodistribution in heart, higher cardioprotective activity of liposomes than that of solution may be expected in further studies on pharmacodynamics.

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***Declaration of interest*:** The authors report no conflicts of interest.

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