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Preparation and Pharmacokinetics in Rabbits of Breviscapine Unilamellar Vesicles

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ABSTRACT The purpose of the study was to prepare the unilamellar liposomal vesicles of breviscapine (Breviscapine-LUVs) and investigate the pharmacokinetics of Breviscapine-LUVs in rabbits. Breviscapine-LUVs were prepared by the film dispersion method and treated further by extrusion. Its size distribution and zeta potential were determined by photon correlation spectroscopy. The encapsulation efficiency (EE) and cumulative release of Breviscapine-LUVs were assayed by the dialysis method. The crossover design (two periods) was used in six rabbits, which were administered Breviscapine-LUVs and reference preparation. Results showed that the particle size of Breviscapine-LUVs was 50.8 nm, and the polydispersity index was 0.287. The zata potential was –24 mV ± 9 mV(n = 3), and the EE% was 81.1 ± 1.1% (n = 3). The cumulative release of vesicles in 0.9% NaCl was 17.2 ± 0.78%, 26.1 ± 0.68%, and 29.9 ± 0.81% in 2, 8, and 24 h, respectively. The mean concentration-time curves of breviscapine liposomes and reference preparation were both fitted to a two-compartment model with the main pharmacokinetic parameters as follows: $t_{1/2\beta}$ of Breviscapine-LUVs and reference preparation were (42.5 ± 28.6) min and (6.01 ± 4.64) min, respectively; $CL_{(s)}$ were (15.3 ± 9.03) mL × min^{-1} and (84.6 ± 40.6) mL × min^{-1}, respectively; AUC_{0-300} were (1267 ± 1083) μg × min × mL^{-1} and (196 ± 107) μg × min × mL^{-1}, respectively. Compared with the reference preparation, breviscapine liposomes had a much higher concentration in plasma and contained characteristic of sustained-release, which ameliorated the pharmacokinetic properties of scutellarin.

KEYWORDS Breviscapine, Scutellarin, Liposomes, Vesicles, Dialysis, Phamacokinetics

INTRODUCTION

Breviscapine is a flavonoid compound derived from the traditional Chinese herb *Erigeron breviscapus*(Vant.) Hand-Mazz. It is extensively used as a natural drug for the treatment of cerebrovascular diseases such as hemiplegia, coronary heart disease, and hypertension (Zhang et al., 2002; Ye & Dong, 1999). The major active compound of breviscapine is scutellarin, a flavone glycoside

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of 4′,5,6-trihydroxy flavone-7-O-β-D-glucuronide (Qu et al., 2001). Its structure is shown as Fig. 1. 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 & Dong, 1998). In recent years, many studies have provided evidence for the neuroprotective effects of scutellarin (Liu et al., 2003a; Hao et al., 2004). It was reported that the concentration of scutellarin in plasma declined rapidly after a single dose i.v. in rabbits (Liu et al., 2003b). The study of biodistribution in mice 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). The metabolism of scutellarin has also been investigated in rats after oral administration. Scutellarin metabolized at a high rate, and five metabolites were detected and identified (Liu et al., 2001).

Liposomes are well-recognized drug delivery vehicles and have been used in drug solubilization, controlled release, and site-specific targeting. Unilamellar liposomes are taken up much less by the reticuloendothelial system (RES) and have long apparent circulating half-time in plasma. Larger liposomes and multilamellar liposomes are rapidly removed from the circulation and localize in the liver and spleen. Thus, unilamellar liposomal vesicles can be designed to prolong drug residence time in vivo. Moreover, liposomes can be stored in a lyophilized state, which improves shelf-life stability of liposomes used as drug carriers.

In this study, to prolong circulating time of breviscapine and delivery breviscapine to cerebrum, we prepared the unilamellar liposomal vesicles of breviscapine (Breviscapine-LUVs) and evaluated their pharmacokinetics in rabbits.

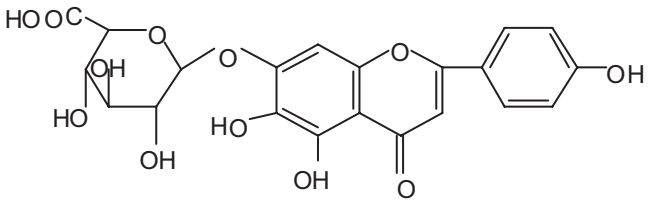

FIGURE 1 **Molecular structure of scutellarin.**

MATERIALS AND METHODS

Materials

Soybean lipid (purity > 92%, lot number 030221) was purchased from Shanghai Taiwei Pharmaceutical Limited Corporation (China), and lecithin was freshly prepared by column chromatography on aluminum oxide according to the established methods. Only lecithin with purity greater than 95% was used in this study. Breviscapine powder (purity > 90%) was supplied by Yunnan Plant Pharmaceutical Limited Corporation (China). Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Breviscapine injection was purchased from Ha'erbin Shengtai Pharmaceutical Limited Corporation (China); 0.9% sodium chloride injection was purchased from Nanjing Xiaoying Pharmaceutical Manufacturing Factory (China). Methanol, chloroform, sodium chloride, and other reagents were the products of Nanjing Chemical Corporation (China). The chemicals for HPLC were of HPLC grade, and all other chemicals were of analytical grade.

Preparation of Breviscapine Unilamellar Liposomal Vesicle

Orthogonal Experiment Design

The preparation technology can influence the encapsulation efficiency (EE) of liposomes, such as preparation methods, organic solvents, and dissolved phases of drug, etc. To improve the EE, we carried out a 3×2 orthogonal experiment as shown in Table 1.

TABLE 1 **Factors and Levels of Orthogonal Experiments Design**

Factors	Symbol	Levels	
Preparation method	A	A1: film evaporation	A2: reverse evaporation
Organic solvent	B	B1: dichloromethane	B2: alcohol acetate
Dissolved phase of drug	C	C1: organic solvent	C2: aqueous solvent

Measurement of Particle Size and Zeta Potential

The particle size and zeta potential of Breviscapine-LUVs after reconstitution were determined at 25°C by photon correlation spectroscopy instrument (MasterSizer3000, Malvern Instruments Co.). Each sample was diluted 100 times in 0.9% NaCl (pH 7.0).

Determination of Malonaldehyde

Quantities of Malondialdehyde (MDA), the oxidized substance of lecithin, in Breviscapine-LUVs were assayed and calculated by thiobarbituric acid (TBA) fluorescence technique (Zhang et al., 2003) at wavelength of 535 nm.

Calculation of Loading Amount of Breviscapine-LUVs

The loading amounts of Breviscapine-LUVs after reconstitution were calculated according to the equation of Loading% = $(W_t - W_i)/W_c \times 100\%$. The W_t was the total amount of breviscapine in liposomes, W_i was the amount of free breviscapine, and W_c was the quantity of lecithin. W_t was determined by HPLC, and W_i was analyzed by dialysis.

Measurement of Encapsulation Efficiency and Cumulative Release

The unencapsulation efficiency of Breviscapine-LUVs (uEE) after reconstitution was calculated as the proportion of the amount of free breviscapine in dialyzing solvent against the total breviscapine in 0.5 mL of liposome suspension. Then the encapsulation efficiency (EE) was calculated according to the equation of EE = 1–uEE. In the same way, the cumulative release of Breviscapine-LUVs was calculated at the predetermined times as the dialyzing continued.

High-Performance Liquid Chromatography (HPLC) Assay Development

The concentrations of breviscapine were determined by high performance liquid chromatography (HPLC; Class-vp5.02, Shimadzu Co., Japan) by using Shimpack CLC-ODS-C18 column (150 mm × 6 mm i.d., Shimadzu, Japan) maintained at 30°C, with the UV detector at 334 nm. The mobile phase was composed of 60% (v/v) water and 40% methanol and delivered at a flow rate of 1.0 mL × min^{-1}. The injection volume was 50 μL and the relative time (t_R) was 10 min.

Pharmacokinetic Studies

Pharmacokinetics of Breviscapine-LUVs After Intravenous Injection in Rabbits

Six New Zealand white rabbits were provided from the Central Animal Laboratory of China Pharmaceutical University. Six New Zealand white rabbits (1.87 ± 0.058 kg) were randomly divided into A and B groups. According to the crossover design, groups A and B received, respectively, commercial breviscapine injection and Breviscapine-LUVs (5 mg × kg^{-1} body weight) via auricle vein of the left ears. Blood samples (1 mL) were taken from the right ears at just prior to and 2, 4, 6, 8, 10, 15, 20, 25, 30, 60, 180, and 300 min following injection. After a 1-week washup period, group A was given Breviscapine-LUVs (5 mg × kg^{-1} body weight), and group B was given commercial injection at the same dosage and blood samples were taken as scheduled.

Plasma sample (0.5 mL) was extracted with 3 mL of ethyl acetate after being acidificated by 100 μL of HCL (0.01 M). After centrifugation for 10 min at 3000 rpm, the organic phase was transferred to another tube and evaporated under a stream of nitrogen at 50°C. The residues were redissolved in 200 μL of mobile phase, and an aliquot of 50 μL was injected for the HPLC analysis. The pharmacokinetic parameters were analyzed by PKANALYSIS program.

RESULTS AND DISCUSSIONS

Preparation and Properties of Breviscapine Unilamellar Liposomal Vesicle

Optimal Preparation Method

The results of orthogonal experiments on liposome preparation were listed in Tables 2 and 3. It showed that the optimal preparation was $A_1B_1C_1$ (A_1: film dispersion method; B_1: dichloromethane; C_1: drug organic solution). Accordingly, appropriate amount of lecithin (187.5 mg), cholesterol (93.8 mg), and breviscapine (37.5 mg) were dissolved in 30 mL of

TABLE 2 Results and Analysis of Orthogonal Experiments Design

No.	A	B	A × B	C	A × C		EE%
1	1	1	1	1	1	1	81.4
2	1	1	1	2	2	2	82.1
3	1	2	2	1	1	2	64.2
4	1	2	2	2	2	1	66.3
5	2	1	2	1	2	1	65.3
6	2	1	2	2	1	2	63.1
7	2	2	1	1	2	2	70.1
8	2	2	1	2	1	1	72.3
K2	270.8	291.9	305.9	218.9	281	285.3	
K1	294	272.9	258.9	345.9	283.8	279.5	
R	–23.2	19	47	–127	–2.8	5.8	
D	–5.8	4.75	11.75	–31.75	–0.7	1.45	

TABLE 3 Analysis of Varian

Source	SS	Rf	ANOVA	F	P
A	67.28	1	67.28	16	>0.05
B	45.13	1	45.13	10.73	>0.05
A × B	276.13	1	276.13	65.67	<0.05
C	2016.13	1	2016.13	479.46	<0.01
A × C	0.98	1	0.98	0.23	>0.05
Error	4.21	1	4.21		

*$F_{0.05}$ = 18.5, $F_{0.01}$ = 98.5.

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 (ALPHA1–2, MARTIN CHRIST Co., Germany).

Particle Size and Zeta Potential of Breviscapine-LUVs

When the lyophilized powder was reconstituted, the particle size of liposome was determined to be 50 ± 12 nm (n = 3), and the polydispersity index was 0.287. The zeta potential was –24 ± 9 mV (n = 3). To deliver drugs intravenously by particles, such as liposomes, it was important to control the particle size. The particle size must be smaller than 5 μm to ensure the safety of administration. Negatively charged liposomes exhibit effectively less uptake by the fixed macrophages of RES, which is related to the circulation half-life ($t_{1/2}$) of the liposomes (Chom et al., 1992).

Stability of Lecithin in the Liposomes

It was reported that hemolysis would occur when the level of MDA in lecithin exceeded 0.1 μg × mg^{-1} (Ulmius et al., 1982). The Breviscapine-LUVs were prepared by the film dispersion method and treated further by extrusion. The mean concentrations of MDA in lecithin before and after preparation of liposome were 0.024 μg and 0.031 μg × mg^{-1} lecithin, respectively. The results showed that the preparation method was good for the stability of lecithin.

Encapsulation Efficiency and Cumulative Release of Breviscapine-LUVs After Storage

For the determination of encapsulation efficiency, the optimal conditions of dialyzing experiment have been studied. It is well known that the rate of dialysis fit for Noyes-Whitney equation: $dC/dt = D \times A \times C_0/(V \times h)$ and was affected by temperature (T), concentration of breviscapine in inner dialyzer (C_0), volume of outer solution (V), and the existence of empty liposome, etc. It is important to make clear that the rate of dialysis is proportioned to the T_{max} of dialysis, the time at which the dialysis equilibrium of the free drug reached. The results listed in Fig. 2 showed that 1) the higher the temperature, the shorter the T_{max}; 2) the C_0 had little influence on the T_{max}; 3) the V had less effect; and 4) the existence of empty liposome did not

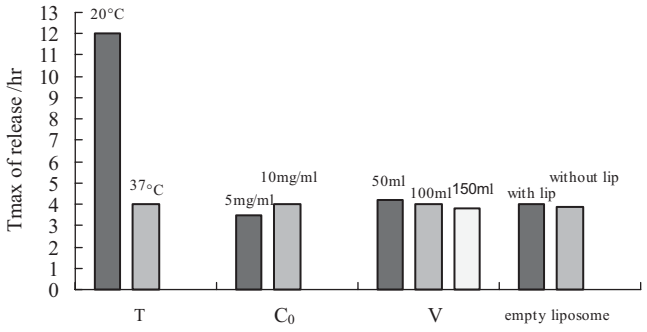

FIGURE 2 **Studies on the factors of dialysis (n = 3). T, temperature; C_0, starting concentration of free drug in inner dialyzer; V, volume of outer solution; empty liposome, the existence of empty liposome.**

hinder the free breviscapine in releasing from the inner dialyzer. According to the optimal conditions, the dialysis was carried out in water bath at 37°C, by using 0.5 mL breviscapine solution (10 mg/mL) as inner solution and 100 mL 0.9% NaCl as outer solution respectively.

The dialysis results of breviscapine were shown in Fig. 3; 98.9% ± 0.05% of breviscapine in the solution could completely permeate the dialyzer within 4 h, which suggested that the free breviscapine can be separated completely from Breviscapine-LUVs by dialyzing for 4 h. According to the results, the EE of breviscapine liposomes was 81.1 ± 1.1% (n = 3), and the loading amount of breviscapine liposomes was 20.1 ± 0.88% (n = 3). The release rates were relatively high in the first 2 h and then began to slow down from 2 to 8 h and turned to be moderate after 8 h. The cumulative release of the liposome in 100 mL 0.9% NaCl was 17.2 ± 0.78%, 26.1 ± 0.68%, and 29.9 ± 0.81% in 2, 8, and 24 h, respectively.

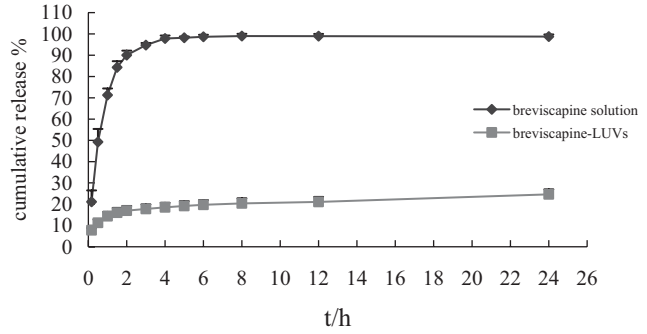

FIGURE 3 **Results of cumulative release of Breviscapine-LUVs (n = 3). ♦, breviscapine solution; ■, breviscapine LUVs.**

HPLC Assay

The regression equation for breviscapine concentrations ($\mu g \times mL^{-1}$) in mobile phase ranged from 0.08 to 20 $\mu g \times mL^{-1}$ was A = 126862C + 7477.3 ($r^2 = 0.9999$, n = 5). The mean recovery was 96.3 ± 0.8% at 5 $\mu g \times mL^{-1}$ (n = 5). Precision assay showed the average of the relative standard deviations (RSD) within 1 day was 1.39% and between days was 1.81%. The content of Breviscapine-LUVs after reconstitution was 9.12 ± 0.02 mg/mL (n = 3).

The regression equation for breviscapine concentrations ($\mu g \times mL^{-1}$) in rabbit plasma ranging from 0.02 to 20 $\mu g \times mL^{-1}$ was A = 224978C – 14110, $R^2 = 0.9993$ (n = 5). The mean recovery was (94.86 ± 1.32)% (n = 3), and RSD values were below 4% within day and 8% between days. The mean extraction recovery was (77.88 ± 0.95)%. The method was precise and reproducible.

Pharmacokinetics Studies

The plasma concentration-time profile of Breviscapine-LUVs was shown in Fig. 4. The profile of commercial breviscapine injection was used as a control. Both curves fit the open two-compartment model by PKANALYSIS program. The pharmacokinetic parameters were listed in Table 4. The $t_{1/2\alpha}$ of Breviscapine-LUVs and commercial injections were (0.603 ± 0.685) and (1.24 ± 0.574) min, $t_{1/2\beta}$ were (42.5 ± 28.6) and (6.01 ± 4.64) min, MRT were (42.8 ± 17.6) and (3.17 ± 1.11) min, AUC were (1267 ± 1083) and (3.17 ± 1.11) $\mu g \times min \times mL^{-1}$, respectively. The mean AUC ratio

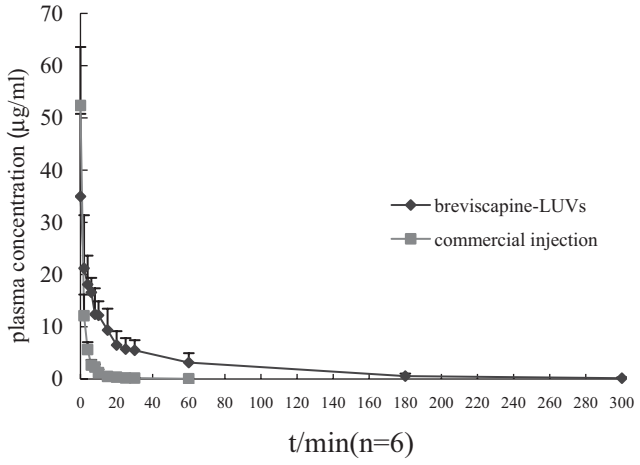

FIGURE 4 **Mean plasma concentration-time profile in rabbits after intravenous injection of Breviscapine-LUVs and breviscapine commercial injections in rabbits (n = 6).**

TABLE 4 **Pharmacokinetic Parameters (n = 6)**

Parameters	Breviscapine-LUVs (mean ± SD)	Commercial injection (mean ± SD)
α/min^{-1}	0.603 ± 0.685	1.24 ± 0.574
β/min^{-1}	0.0224 ± 0.0158	0.153 ± 0.0627
$t_{1/2\alpha}$/min	3.59 ± 3.52	0.675 ± 0.314
$t_{1/2\beta}$/min	42.5 ± 28.6	6.01 ± 4.64
$CL_{(s)}$/mg/kg/min/(ug/ml)	15.3 ± 9.03	84.6 ± 40.6
AUC/$\mu g \times min \times mL^{-1}$	1267 ± 1083	196 ± 107
MRT/min	42.8 ± 17.6	3.17 ± 1.11
$V_{(c)}$/mL	248 ± 199	152 ± 134

of Breviscapine-LUVs to commercial breviscapine injection was 646%.

The pharmacokinetic parameters of commercial breviscapine injection showed that breviscapine was rapidly cleared in the bloodstream. The $t_{1/2\beta}$ was only (6.0110 ± 4.6397) min. Such results were consistent with the analysis of ^{3}H-scutellarin (Cai, 1981), in which the large amount of radioactivities was recovered in cholecyst and intestine after i.v. 1 h.

The pharmacokinetic parameters of Breviscapine-LUVs showed that the liposome did increase the breviscapine concentrations in plasma, retarded the clearance, and exhibited the properties of sustained-release. To interpret the biopharmaceutic behavior of breviscapine entrapped in liposomes, the studies on tissue distribution of Breviscapine-LUVs would be carried out in future.

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