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PREPARATION OF SCUTELLARIN LOADED TPGS POLYMERIC MICELLES AND EVALUATION OF ITS PHARMACOKINETICS AND PHARMACODYNAMICS EFFECTS IN RATS

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Abstract: To improve the clinical effect of scutellarin by extending the action time *in vivo*, scutellarin loaded polymeric micelles were developed by D-alpha tocopherol polyethylene glycol 1000 succinate (Scu/TPGS). Scu/TPGS were prepared using film solvent diffusion methods and characterized on the basis of their particle size, zeta potential, and drug encapsulation efficiency. Dynamic dialysis was used to study the release behavior of the polymeric micelles *in vitro*. Its pharmacokinetic characteristics and antithrombotic efficacy were studied by intravenous injection in rats. The results showed that Scu/TPGS were spherical, 20.09 ± 2.62 nm in size and a slow release *in vitro*. The pharmacokinetic parameter $T_{1/2}$ of Scu/TPGS was 762.12 ± 46.56 min compared with commercial injection of 59.30 ± 10.67 min ($p < 0.05$). At the 1 mg/kg dose, the thrombolysis effect of micellar group was stronger than that of the commercial group ($p < 0.05$). In conclusion, TPGS polymer micelles provided a valid strategy in chemotherapy for cerebrovascular diseases with poor water solubility and poor lipid solubility drugs such as scutellarin.

Keywords: scutellarin, TPGS, polymeric micelles, antithrombotic drugs

Breviscapine is a genus of plant flavonoids in Erigeron breviscapus extracted from Chinese herb of Erigeron breviscapus (vant) Hand–Mazz, the main component of which is scutellarin (4',5,6-trihydroxyflavone-7-O-glucuronide) (1). Breviscapine has been made into some Chinese patent medicines including injections and tablets and for the treatment of cardiovascular and cerebrovascular diseases in clinical practices (2). However, scutellarin has low water-solubility (just 14.4 μg/mL) and lipid-solubility (log P = -2.56 in PBS at pH 4.2) (3), and the bioavailability of scutellarin is very low with the absolute oral bioavailability in Beagle dog rarely 0.4% (4). What's more, the breviscapine has a short residence time in the circulation (5). The pharmacokinetics of breviscapine at a single intravenous dose to rabbits and dogs showed that scutellarin was very fast and rapidly eliminated from the blood. The $T_{1/2}$ of scutellarin was (12.0 ± 6.6) min and (6.99 ± 2.74) min in rabbits and dogs respectively (6, 7). The ischemic cerebrovascular and cardiovascular diseases dealt with breviscapine are chronic and always require prolonged treatment times.

In order to overcome the above defects of scutellarin, many approaches have been experimented. For example, Lu et al. (8) systematically synthesized the PEG-scutellarin prodrug by linking the difference molecular weight of mPEGs to difference reactive sites of scutellarin to elucidate the effect of PEGylation of scutellarin on solubility, activity, and half-life. Jiang and Yang et al. (9, 10) synthesized the scutellarin-cyclodextrin conjugates which provided a useful approach to develop a highly effective drug candidate for human colon cancer chemotherapies. Xiong et al. (11) used a series of MO–PEG–COOH to modify the lipid emulsions surface for carrying breviscapine, which might enhance the pharmacological activity of breviscapine to promote blood circulation. Zhong and Lv et al. (12, 13) studied that breviscapine liposomes delivered more scutellarin

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into the plasma than the breviscapine solution. He et al. (14, 15) made sustained and controlled release preparation of breviscapine.

The process of exocytosis of flavonoid drugs which was shown to be a substrate of both MRP1 and MRP2 (16) among those drug delivery strategies were not discussed. D-alpha tocopherol PEG 1000 succinate (TPGS) has an effect on inhibiting P-gp mediated multidrug resistance, thus inhibiting exocytosis of flavonoid drugs, enhancing the absorption of drugs. TPGS has been widely used as a modifier of polymeric micelles in many researches and has an effect on inhibiting P-gp mediated multidrug resistance, thus inhibiting exocytosis of flavonoid drugs, enhancing the absorption of drugs (17, 18).

TPGS has an amphiphilic structure composed of a hydrophilic group (PEG) and a lipophilic group (tocopherol succinate). Due to its favorable hydrophilic-lipophilic balance (HLB) value, TPGS is an excellent hydrophobic drugs emulsifier, solubilizer, and bioavailability enhancer (19, 20). Furthermore, TPGS has an effect on inhibiting P-gp mediated multidrug resistance, enhancing the absorption of drugs. TPGS has been approved by the FDA as a safe pharmaceutical adjuvant for pharmaceutical formulation (21-23).

We prepared scutellarin loaded TPGS polymeric micelles (Scu/TPGS) by the film solvent diffusion method (Scheme 1). Polymeric micelles can improve the ability of the drug membrane permeability and stability, reduce toxicity and improve the bioavailability of the drug *in vivo* (24). The surface of polymeric micelles modified by polyethylene glycol (PEG) has been widely studied because of prolonging the circulation in blood besides the above advantages (25). In this study, the micelle's size distribution, surface morphology, drug encapsulation efficiency and drug release profile were evaluated. The intravenous of Scu/TPGS was evaluated in rats. Moreover, the thrombosis inhibition effect of Scu/TPGS was assessed in thrombosis mice.

MATERIALS AND METHODS

Breviscapine (Scutellarin of 95% purity) and Scutellarin powder injection was provided by Hunan Heng Sheng pharmaceutical Co.Ltd. (Hengyang, China). Scutellarin standard (purity > 98%) and rutin (internal standard, IS) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). TPGS was purchased from Aladdin (China). Pentobarbital (China) and Cellulose ester membranes with a molecular weight cut-off value (MWCO) of 3500 (Greenbird Inc., Shanghai, China) are used in dialysis study. Other reagents and chemicals were commercially available and of high-performance liquid chromatography (HPLC) and analytical grades.

Animals

Wistar rats (body weight of 200 ± 20 g), were obtained from Experimental Animal Center of University of South China. Animals were in the environment with a temperature of 24 ± 1°C and humidity of 65%-70%, and were placed on a 12 h light/dark cycle, and allowed free access to standard laboratory chow and tap water.

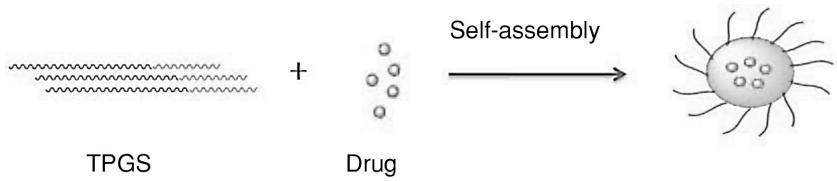
The protocol of the study was approved by The Institutional Animal Care and Committee of the University of South China (approval number: SYXK (Xiang) 2015-0001). The Declaration of Helsinki as amended in Seoul 2008 for humans, and the European Community guidelines as accepted principles for the use of experimental animals, were adhered to.

Chromatographic conditions

The concentration of scutellarin was detected by HPLC. The HPLC system has consisted of four pumps (HP1260, Agilent, USA), a C_{18} column (4.6 mm × 250 mm, 5 µm, Agilent, USA) maintained at 30°C. The mobile phase consisted of acetonitrile – 0.1% phosphoric acid (pH 3.0, 27 : 73 v/v) was delivered at a flow rate of 0.7 mL/min. We did the testing at a wavelength of 335 nm, and the injection volume for samples was 20 µL.

Preparation of Scu/TPGS

Scu/TPGS were made by the film solvent diffusion method (17). Briefly, TPGS (100 mg) and scutellarin (7 mg) were co-dissolved in methanol solution. Then the solution was removed by the



Scheme 1. The scheme of scutellarin loaded TPGS polymeric micelles

rotary vacuum evaporation. The formed film was additionally hydrated with 10 mL distilled water, incubated at 37°C for 3 h. Then, add some methanol solution, the solution obtained was to be removed organic solvents by rotary vacuum evaporation. Follow the methods mentioned above and repeat 3 times. Finally, the mixture solution was filtered by 0.45 μm polyethersulfone syringe filter in a sterile environment and then micelles were triumphantly prepared. The blank micelles were prepared following the method above except that the scutellarin are not added.

Particle size, polydispersity, and zeta potential

The index of particle size and zeta potential of the polymeric micelles were assessed by dynamic light scattering (DLS) using a Zeta sizer Nano ZS instrument (Malvern Instruments, Malvern, UK). Each experiment was carried out in triplicate.

Morphological characterization

The morphology of the polymeric micelles was observed using H-7650 TEM (Hitachi, Tokyo, Japan) at an accelerating voltage of 80 kV. Micelles (1 mg/mL) were attenuated with distilled water and placed on a copper mesh and left to adhere for 2 min, and filter paper was used to wipe off the excessive liquid on the mesh edges. Freshly prepared 2% sterile phosphorus tungstate solution was added for negative staining. The negative dye was then absorbed. The copper wire mesh was dried at room temperature and placed under the electron microscope and photographed to observe micelles morphology.

Drug loading and encapsulation efficiency

The scutellarin encapsulation efficiency of Scu/TPGS was determined by UV spectrophotometer (Shimadzu 1750, Tokyo, Japan). Scu/TPGS for 1 mL was diluted with 20 mL methanol and sonicated for 10 min with a KQ-5200 Ultrasound Sonicator (Kunshan Ultrasound Instrument Co., Ltd., Kunshan, China). The entrapment efficiency (EE) and drug loading (DL) were calculated using the following equations:

$$EE\% = \frac{\text{Weight of scutellarin in micelles}}{\text{Weight of scutellarin added initially}} \times 100\%$$

$$DL\% = \frac{\text{Weight of scutellarin in micelles}}{\text{Weight of polymeric micelles}} \times 100\%$$

***In vitro* drug release**

Dialysis bag diffusion technique was used to study the release patterns of scutellarin from Scu/TPGS and free scutellarin (26, 27). The formulation of a volume equivalent to 1 mg scutellarin was placed in the dialysis bag which was hermetically sealed and immersed in 50 mL phosphate buffered saline (PBS, pH 7.4) containing 0.2% EDTA-2Na at 37 ± 0.5°C, with rotation at 100 rpm. Then, an aliquot of 1 mL samples were taken out from the receptor compartment at pre-determined time intervals (0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12, 24, 36 and 48 h) and mixed with equal volume of fresh medium. The content of scutellarin in the samples was detected by UV spectrophotometer. Then drug release profiles were countered (28, 29). The calculation equation for the drug release curve was $RR = (W_i/W_t) \times 100\%$, where W_i is the quantity of scutellarin released at different time points and W_t is the total quantity of scutellarin added before the release started. We used the indexes of drug release and standard deviation (mean ± SD, n = 3) to plot the release profiles. The test was carried out in triplicate.

***In vivo* pharmacokinetic studies**

Twelve rats were randomly separated into two groups and fasted for 12 h except for free access to water for pharmacokinetics studies (30). After the intravenous injection of Scu/TPGS and commercial injection (equivalent to 10 mg/kg of scutellarin), 250 μL of blood were collected from orbital vein of rats and placed in heparinized tubes at different time points (0.5, 1, 2, 4, 6, 8, 12 and 24 h). The gathered blood was centrifuged at 4000 rpm (centrifuge 5810R, Eppendorf, Germany) for 15 min. All plasma samples were stored at -20°C for further treatment.

Plasma samples preparation

We used the protein precipitation method to determine the content of scutellarin. 100 μL plasma sample, 20 μL internal standard solution (100 g/mL rutin in methanol) and 20 μL L mol phosphoric acid, and 20 μL 0.2% EDTA-2Na were added and blended for a few seconds. Then proteins were precipitated after 1.0 mL methanol being added. After the samples were vortex mixed for 5 min, the compound was followed by centrifugation at 10000 rpm for 5 min. The supernatant was transferred to a fresh tube and the lower precipitation was added 0.5 mL methanol and shaken for 5 min on a horizontal shaker, followed by centrifugation at 10000 rpm for 5 min. Then, draw on the clear liquid, and merge the two clear liquid. The organic horizon was transferred to another tube and the solution was evaporated to dryness in a water bath at 40°C through a stream of nitrogen. The residue was dissolved in 100 μL methanol and 20 μL was applied to the HPLC analysis.

***In vivo* thrombolysis efficacy**

24 rats were randomly separated into six groups, which contained control group (establishment of thrombus model with saline), model group (physiological saline solution), commercial injection group, low dose of micelles group (Micelles-LD), middle dose of micelles group (Micelles-MD) and high dose of micelles group (Micelles-HD), respectively. In a rat model of carotid artery thrombosis induced by local ferric chloride, an increase in transient blood flow velocity (VEL) was observed immediately after administration of ferric chloride application. After carotid midline incision, the left common carotid artery (LCCF) was exposed carefully. 1% pentobarbital sodium was used to anesthesia rats (5 mL/kg). About 2 cm rat's left carotid artery was isolated. Afterward, the plastic film (4 cm × 2 cm) was placed under carotid artery to protect the surrounding vascular tissue. A filter paper disk (diameter 1 cm) saturated with 35% (w/v) ferric chloride solution was placed on the surface of the carotid artery for 30 min. The wound was sutured after washing with saline. After rats awakened, we gave them injection for three days consecutively. At last, we anesthetized rats again and remove the left thrombus and isometric contralateral normal blood vessels. Put them in methanol solution for fixation and then dry at 60°C for 2 h. Remove the blood vessels from methanol and then scaled the weight of its thrombus with an electronic analytical balance. The subtraction before and after is for 1 cm length of the thrombosis quality of blood vessels. The thrombolysis rate of each group was calculated according to the formula below, where Mi is the thrombus weight of model group and Mj is the thrombosis weight of the administration group.

Statistical analysis

All experiments results are presented as the mean ± standard deviation (SD). The data were analyzed by SPSS software (version 17.0; SPSS Inc., Chicago, IL, USA). The statistical significance of differences was evaluated by two-way ANOVA analysis. $P<0.05$ was considered as statistically significant.

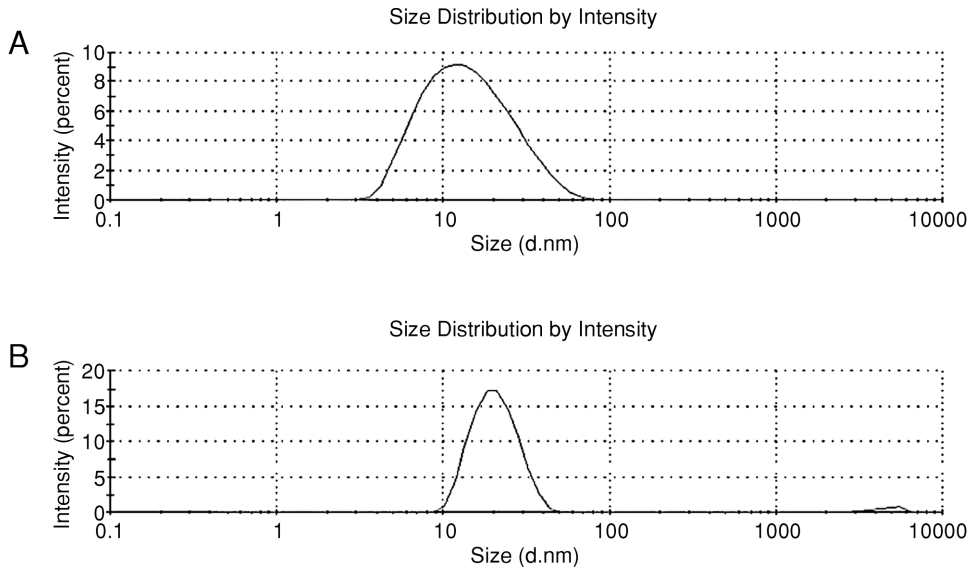


Figure 1. Particle size distribution of blank micelles (A) and Scu/TPGS (B)

Table 1. Particle size, polydispersity, encapsulation efficiency, drug loading of blank micelles and Scu/TPGS (n = 3).

	Particle size(nm)	PDI	EE%	DL%
Blank micelles	12.14 ± 0.39	0.218 ± 0.04	-	-
Scu/TPGS	20.09 ± 2.62	0.177 ± 0.01	90.18 ± 2.14	5.83 ± 0.19

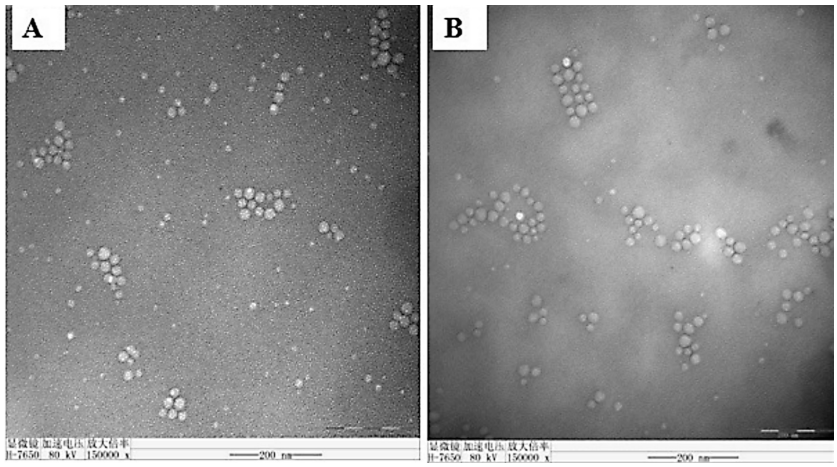


Figure 2. TEM image of blank micelles (A) and Scu/TPGS (B)

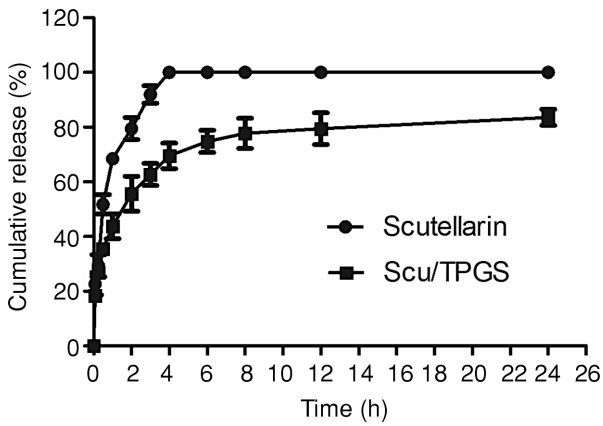


Figure 3. *In vitro* profiles of scutellarin and Scu/TPGS in PBS (pH 7.4) with 0.2% EDTA-2Na (w/v) at 37°C. Indicated values were mean ± SD (n = 3)

RESULTS AND DISCUSSION

Preparation and characterization of Scu/TPGS

The characteristics of blank micelles and Scu/TPGS including particle size, PDI, EE, and DL were shown in Table 1. The distribution curves of particle size were unimodal for all the samples. The mean sizes of blank micelles and Scu/TPGS were 12.14 ± 0.39 nm and 20.09 ± 2.62 nm respectively (Fig. 1). The results of particle size indicated that Scu/TPGS led to a significant ($p < 0.05$) increase compared to blank micelles. The size of Scu/TPGS was bigger than blank micelles. The results might reveal that the encapsulated micelles contained more drugs in their cores, thereby increasing the diameter of the aggregated micelles. All the polymeric micelles polydispersity showed a quite narrow size distribution, which were near to 0.2. The zeta potential was a measure of the intensity of the repulsion or attraction between the particles. The smaller the molecular or dispersed particles and the higher the zeta potential (positive or negative) were, the more stable the system was. That is, the dissolution or dispersion can resist aggregation. On the contrary, the lower the zeta potential (positive or negative) was, the more inclined to condense or agglomeration. That is, the attraction exceeds the repulsive force, and the dispersion is destroyed thus coagulates or condenses. The zeta potential of Scu/TPGS was about 0 mV. It was mainly due to the hydrophilic polyethylene glycol (PEG) chains could form a neutral PEG hydration layer in the outer layers, thus reducing the electronegativity. So the micelle was not easy to be recognized and swallowed up by the reticuloendothelial system (RES), thus extending the time of drug in blood circulation (31). The nanomicelles could avoid a meshy endodermis system being gobbled up and prolong the blood circulate

time (32, 33). The TEM results (Fig. 2) showed that blank micelles and Scu/TPGS displayed a uniform spherical morphology.

***In vitro* drug release**

Temperature and pH had a significant effect on the stability of the aqueous solution of scutellarin. Its stability decreased with the increase of temperature and pH. It was found that EDTA-2Na significantly improved the stability of scutellarin (34). Therefore, pH 7.4 phosphate buffers with 0.2% EDTA-2Na was used as the release medium on the basis of references and preliminary experiment. The experiment was performed to assess the profile of drug release from the Scu/TPGS and free scutellarin, respectively. As shown in Figure 3, the Scu/TPGS exhibited a sustained-release profile compared with scutellarin in a dispersion medium. The percentage of free scutellarin's cumulative release was 100% within 4 h. The results showed that scutellarin could enter into the dissolution medium entirely in time through the dialysis membrane and scutellarin could be released slowly from micelles also (35, 36).

***In vivo* pharmacokinetics studies**

After i.v. administration of 10 mg/kg equivalent scutellarin, we set up the rat plasma sample analysis method with scutellarin, rutin as an internal standard. Scutellarin and rutin had a good degree of separation, and endogenous substances to be determined without interference. It had proved that the method of precision, repeatability and recovery rate were in line with the requirements of biological sample analysis. The plasma concentration-time graphs of scutellarin in rats were shown in Fig. 4 and the pharmacokinetic parameters are listed in Table 2. The $AUC_{0\text{-inf}}$ (the area under plasma concentration-time curve) and $MRT_{0\text{-inf}}$ (mean residence time) of Scu/TPGS were 491.01 ± 74.23 min and 1474.76 ± 35.79 min, which were 1.67-fold and 22.7-fold higher than those of commercial injection, respectively. More importantly, the commercial injection was eliminated nearly in 4 h. However, the micelles could significantly prolong its $T_{1/2}$ (half-life) that confirmed the lasting circulation potential. This might due to the presence of PEG at micelles' surface. TPGS prevented adhesion of plasma proteins (by hiding properties), thus avoiding recognition by the reticuloendothelial system, which absolutely improved systemic circulation time. These results suggested that commercial injection and Scu/TPGS were undergoing extensive binding with tissue. Mean residence time (MRT) of Scu/TPGS was 22.7 times higher than commercial injection (37). The overall observation of higher AUC, $T_{1/2}$, and MRT of Scu/TPGS evidently signified that the present polymeric micelles would be a potential compound for improving systemic availability and extending the systemic circulation of scutellarin. As a result of small particle size of Scu/TPGS and protection by hydration physical barrier layer formed by PEG chain, the micelle was not easy to be recognized and swallowed up by the reticuloendothelial system (RES) and or discharged by the liver, spleen and so on. They prolonged the cycle time and kept stable of drug in the body. The drug had a longer retention time in the blood circulation.

Carotid artery thrombolysis experiment

A thrombus was a blood clot that forms in blood vessels and interrupts blood circulation. A thrombus may cause cardiovascular or cerebrovas-

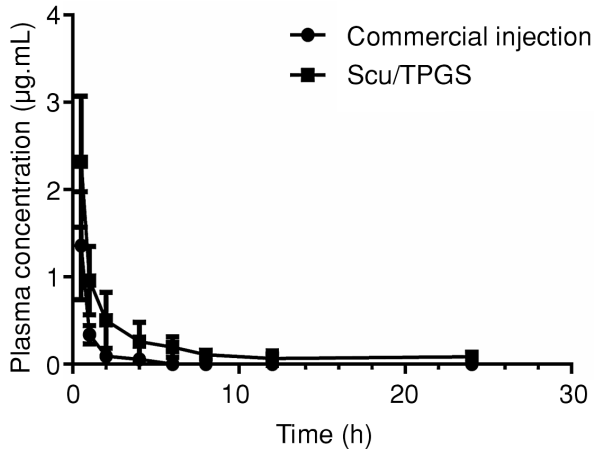


Figure 4. Mean plasma concentration profile in rats after intravenous injection of Scu/TPGS and commercial injection up to 24 h. Indicated values were mean ± SD (n = 6)

Table 2. Plasma pharmacokinetic parameters of scutellarin after i.v. commercial injection and Scu/TPGS in rats (n = 6).

Pharmacokinetic parameters	Commercial injection	Scu/TPGS
$T_{1/2}$(min)	59.30 ± 10.67	762.12 ± 46.56*
T_{max}(min)	5.00 ± 0	5.00 ± 0
C_{max}(μg/mL)	15.71 ± 2.11	9.53 ± 1.64
$MRT_{0\text{-}inf}$(min)	66.73 ± 10.35	1474.76 ± 35.79*
$AUC_{0\text{-}inf}$/min*(μg/mL)	293.67 ± 89.62	491.01 ± 74.23*

*$p < 0.05$ vs commercial injection

Table 3. Thrombotic rate and weight of thrombus in different groups (n=4).

Group	Dose	Dry weight(mg)	Ration of thrombotic (%)
Control	-	-	-
Model	-	3.675 ± 0.54	-
Commercial injection	1 mg/kg	2.850 ± 0.17*	22.45 ± 4.71
Micelles-LD	0.5 mg/kg	2.875 ± 0.15*	21.76 ± 1.36
Micelles-MD	1 mg/kg	2.525 ± 0.27*$^{\triangle}$	31.29 ± 3.41
Micelles-HD	2 mg/kg	2.325 ± 0.17*$^{\triangle}$	36.73 ± 4.6

*$p < 0.05$ vs model group; $^{\triangle}p < 0.05$ vs commercial injection

cular diseases such as myocardial infarction, stroke, or cerebral infarction due to the discontinuity in oxygen supply. Compared with the model group, the weight of thrombus in different drugs group was decreased at different level. Thrombus dissolution of the scutellarin group was obvious enhanced. The difference was statistically significant ($p < 0.05$). Compared with the commercial injection, the weight of thrombus in the micellar group was significantly reduced ($p < 0.05$), and the thrombus weight of the drug group was reduced. The thrombolytic rate was significantly greater than the commercial group (Table 3). The micelles of high, medium and low dose groups were in a concentration-dependent manner. Compared with the commercial injection, the thrombolysis rate was also significantly higher in the medium and high dosage of the micellar group than the commercial injection group. The weight of thrombus of the commercial group and low dose of the micellar group was also decreased, but it did not reach a significant difference. The thrombolysis rate was 22.45 ± 4.71% in the commercial group and 21.76 ± 1.36%, 31.22 ± 3.41%, 36.73 ± 4.65%. The thrombolysis rate of micellar group was higher than that of the same dose of scutellarin, suggesting that polymer micelles could improve the therapeutic effect of the scutellarin drug (38, 39).

CONCLUSIONS

In this study, TPGS-coated liposomes were prepared as a drug delivery system to enhance the therapeutic efficacy of scutellarin. The prepared polymeric micelle was shown in a spherical shape with small size. The drug release test revealed *in vitro* that Scu/TPGS represented a sustained release profile. Pharmacokinetic experiments demonstrated that scutellarin polymer micelles had better behavior which showed a significant increase in $T_{1/2}$, MRT, and AUC. It could be observed that the bioavailability of Scu/TPGS was greatly improved in comparison to commercial injection. *In vivo* thrombolysis test also confirmed that thrombolytic effect of the polymer micelles was stronger than the equal dose of commercial scutellarin. In conclusion, Scu/TPGS should be a kind of promising candidate in enhancing the thrombolytic effect of scutellarin.

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