World Journal of Biochemistry and Molecular Biology
2018; 3(1): 16-21
http://www.aascit.org/journal/wjbmb

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Pharmacokinetics and Tissue Distribution of Ligustrazine Phosphate Liposome in Rats

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Citation

Li Siqi, Yi Xuetao, Li Linfeng, Wu Haonan, Li Xuanze, Xin Lili, Zhang Guowei. Pharmacokinetics and Tissue Distribution of Ligustrazine Phosphate Liposome in Rats. *World Journal of Biochemistry and Molecular Biology*. Vol. 3, No. 1, 2018, pp. 16-21.

Received: March 15, 2018; **Accepted**: April 3, 2018; **Published**: May 9, 2018

Abstract: *Objective:* To research the ligustrazine phosphate Liposome bioavailability and brain targeting by study the pharmacokinetics and tissue distribution of ligustrazine phosphate liposome in Rats. *Methods:* 48 Rats were intravenous injected ligustrazine phosphateand its ligustrazine phosphate Liposome. The dose level was 50 mg/kg, after the administration, blood was collected at 0.083h, 0.25h, 0.5h, 1h, 2h, 4h, 5h, at terminal time points, heart, liver, spleen, lung, kidney, and brain were excised. All samples were detected with LC/MS/MS method. Pharmacokinetic parameters were calculated with DAS 2.1.1 software using non-compartmental analysis. *Results:* No significant difference of pharmacokinetics parameters in blood were existed between the reference formulation and lipidosome formulation. The mean AUC 0-∞ (mg/L*min) in heart was 659.653±200.689 for lipidosome formulation while 586.67±46.635 for reference formulation, the difference was statistically significant, obviously, intake of ligustrazine phosphate in lipidosome formulation achieved more in heart. As for plasma distribution, the Tmax of lipidosome formulation was longer than reference formulation, so ligustrazine phosphate was assimilated slowly in lipidosome formulation. The AUC (0-∞) and Cmax were 784.078±196.337 and 13.103±3.216 in lipidosome formulation, respectively. Brain was distributed more when ligustrazine phosphate was in lipidosome formulation. Undisputedly, this act could contribute to the effective treatment. *Conclusion:* The ligustrazine phosphate lipidosome formulation improves the ligustrazine phosphate bioavailability and brain targeting.

Keywords: Ligustrazine Phosphate, Liposome, Rats, Pharmacokinetics, Tissue Distribution

1. Introduction

2,3,5,6-Tetramethylpyrazine (TMP, ligustrazine), a main active compound originaly isolated from Ligusticum wallichii franchat (a Chinese medicine herb, named Chuan Xiong), has been widely used in China for the treatment of ischemic cardiovascular and cerebrovascular disease [1-2], is capable of antioxidation, reduction of calcium overload and inhibition of inflammatory. It has the effects of vasodilatation and antihypertension. It can relieve pain, inhibit inflammation and reduce tissue damage of various diseases. Ligustrazine phosphate intravenous injection was mainly adopted for insufficient cerebral blood flow, cerebral thrombosis and cerebral infarction in clinical therapeutics [3-10]. Short elimination t1/2 and low bioavailability limited its treatment effect. TMP penetrated through the blood–brain barrier within 10 minutes but was eliminated rapidly, 2 hours after administration, plasma level of TMP was undetectable. Similarly, rapid distribution and elimination from brain tissue of TMP were reported after intranasal administration to rats. Liposome technology is reported as a better method to improve the Poor bioavailability and poor pharmacokinetic characteristics. Liposomes promote the rapid transmission of the blood brain barrier by drug [8]. For improve the

ligustrazine phosphate bioavailability and brain targeting, ligustrazine phosphate was designed into liposome to increase the therapeutic concentration, and improve the pharmacokinetics parameters.

2. Materials and Methods

2.1. LC/MS/MS Conditions

The analyses were performed on an Agilent 1290/6460 HPLC-MS equipement, which consisted of a waters sunfire C18 (250*4.6mm, 5μm), the atomizing pressure was 40 psi, the carrier gas N2 was at a rate of 13 L·min-1, 350°C, capillary voltage (4000 V), the positive eletro-spray ionization (ESI+) with selected ion monitoring (SIM), the monitoring ion m/z were 137.1 (Ligustrazine) and 195.1 (Caffeine). The flowing phase was a mixture of methanol (A): 0.05% acetic acid (B). Gradient elution, 0-5 min, A (10% to 10%); 5-14 min, A (10% to 90%); 14-15 min, (90% to 10%); 15 -20 min, A (10% to 10%); all LC/MS/MS measurements were at a flow rate of 1 mL/min for 25 min, the sample size was 10 μL.

2.2. Ligustrazine Phosphate Liposome Preparation and Its Quality Control

TMPP were prepared by melting method. Soyabean lecithin was selected as the phospholipids, with cholesterol was selected as the steroid, and polyethylene glycol 400 was chosen as the surfactant. Soyabean lecithin: cholesterol: water: TMPP: polyethylene glycol 400, five ratios were executed, that's 5/1/5~15/1/0.1~1.5, 4/1/5~15/1/0.1~1.5, 3/1/5~15/1/0.1~1.5, 2/1/5~15/1/0.1~1.5, and 1/1/5~15/1/0.1~1.5. The preparation process was described as below. Firstly, soyabean lecithin and polyethylene glycol 400 were mixed with a little water, and cholesterol was melted into liquid at 200 degrees Centigrade. Phosphate buffer solution (PBS) was prepared at 45 degrees Centigrade, and equal proportion of TMPP was added into the hot PBS solution, this mixed solution was stirred till dissolved completely. Subsequently this mixed solution was added into the solution containing soyabean lecithin and polyethylene glycol 400, the mixed solution was stirred for 30 minutes, and it was added into the liquid cholesterol and stirred for one more 30 minutes. Once the oil phase was mixed with the water phase, the mixture was sheared with high speed shearing machine for one minute, and the revolutions rate was 16000 rpm per minute. Following it, the mixture was stirred with glass rod for one minute. After three cycles of shearing and stirring, the primary emulsion was prepared. At last, the primary emulsion was homogenized by micro-jet method at 10000 psi. After three cycles of homogenous operation, the homogenous liposome solution was prepared successfully. Then 50 mg equivalent Ligustrazine in homogenous liposome solution was added into the vials. Then these were sent to freezing chamber, and all were dried by vacuum drying at -58 degrees centigrade for 30 h. Once finished, all vials were lidded with rubber plug, pressed with aluminium cap and wax-sealed. As for liposome quality control, three inspection items were included, the form and shape, particle size, dissolvability and encapsulation efficiency. The form and shape was observed with transmission electron microscope. The particle size was measured by Dynamic light scattering (DLS), and the polydispersity index (PDI). And 5 dissolvability was detected for the dissolving time. For the encapsulation efficiency calculation, the homogenous liposome solution was filtered via ultrafiltration method, and big particles will be excluded, leaving the liposomes with a molecular weight less than 3000. Then the absorbance of the filter solution was measured by UV spectrophotometry. The encapsulation efficiency (EE) was calculated as below. EE%= (Total drug consumption – The concentration of Ligustrazine×Volume of homogenous liposome solution before ultrafiltration)/Total drug consumption×100.

2.3. Test Animal and House Condition

48 Wistar rats were purchased from Academy of Military Medical Sciences (Beijing, China), half male and half female. All rats were handled following the Guiding Principles for the Care and Use of Experimental Animals from Hebei University and approved by the institutional committee on animal care. After acclimation for a week, they were divided into two groups, the reference group and the liposome group. During the experiment, all were maintained under standard environmental conditions (23±2°C, 55±5% humidity and 12 h/12 h light/dark cycle). Food and water were available ad libitum.

2.4. Drug Administration and Sample Collection

All animals were fasted without food for 12 h before administration. The dose level for all groups was 50 mg/kg, and the dose route was femoral vein. After the administration, blood was collected at 0.083 h, 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 5 h via orbital venous plexus, 0.4 mL blood per time point, and blood was anticoagulated with heparin. Once centrifuged at 5000 r·min-1 for 5 minutes, plasmas were cryopreserved at -20℃ degrees centigrade. At terminal time points, heart, liver, spleen, lung, kidney, and brain were excised. Then all tissues were homogenized with 2-fold 0.9% saline, after 5000r·min-1 centrifugation for 5 min, the supernatant was collected, all samples were stored at -20°C for detection. Sample processing For plasma samples, 50μL plasma was obtained per time point. Each was added 6 with 10 μL methanol, and 10 μL internal standard solution (100.4 μg/mL caffeine solution). After vortex, 150 μL methanol was added to precipitate the protein in samples. Following this, all samples were vortexed and centrifuged for 5 minutes at 11000r/min, the supernatants were detected with LC/MS/MS. And for tissue samples, 50μL homogenizing tissue suspension was prepared per animal organ. Each was added with 10μL methanol, and 10 μL internal standard solution (41.6 μg/mL caffeine solution). Following vortex, 150 μL methanol were added to precipitate the protein in samples. After vortex, all samples were centrifuged for 5 minutes at 11000 r/min, the supernatant were detected with LC/MS/MS.

2.5. Preparation of Calibration Standard in Plasma and Tissue

Standard Ligustrazine Phosphate and caffeine were prepared for stock standard solutions with methanol. For plasma sample, concentrations of the stock solutions were 100.4 μg/mL and 948.861 μg/mL, respectively. Eight concentrations of standard Ligustrazine Phosphate were prepared by dilution of the stock standard solution with blank plasma, it were 0.047, 0.095, 0.474, 1.898, 4.744, 18.977, 37.954, 56.932 μg/mL, the remaining steps would be the same as sample processing. As for tissue sample, concentrations of the stock solutions were 41.6 μg/mL and 1032 μg/mL, respectively. Eight concentrations of standard Ligustrazine Phosphate were prepared by dilution of the stock standard solution with blank tissue relatively, it were 0.052, 0.103, 0.516, 2.064, 5.160, 20.640, 41.280, 61.920 μg/mL, the remaining steps were the same as tissue processing.

2.6. Method Validation Speciality

50 μL blank plasma was processed in the same way as "sample processing", and detected to obtain the blank plasma chromatogram. Plasma solution containing 2μg·mL-1 Ligustrazine Phosphate Liposome was processed in the same way as "sample processing", and detected to obtain the Ligustrazine Phosphate chromatogram containing the caffeine. The reference Ligustrazine Phosphate 7 Injection was administrated at 50 mg/kg, subsequently the plasma was processed in the same way as above, and detected to obtain the Ligustrazine Phosphate chromatogram and the caffeine chromatogram. At last, the relative retention time was 12.3 min and 9.3 min for Ligustrazine Phosphate and Caffeine, respectively. This all indicated that no interference exposed between the two compounds in blank plasma. The Caffeine could be used to detect the content of Ligustrazine Phosphate in plasma or tissue.

2.6.1. Lower Limit of Quantitation

0.05μg·mL-1 Ligustrazine Phosphate was prepared in the same way as the standard curve samples, then it was processed according to the "sample processing", 6 parallel aliquots were detected, the relative standard deviation was 3.24%, the Signal to Noise ratio (SNR) was greater than 10, which all indicated that the Lower limit of quantitation low extended to 0.05μg·mL-1. Recovery 0.6 μg/mL and 2.4 μg/mL standard Ligustrazine Phosphate plasma solution was prepared in the same way as the standard curve samples, 6 parallel aliquots were obtained, after detection, the Peak area of Ligustrazine Phosphate was calculated as At; Two aliquots of solution were prepared in the same way as above, except the blank plasma was replaced with pure water, after detection, the Peak area of Ligustrazine Phosphate was A0, the absolute recovery was calculated from the result of At/A0*100.

2.6.2. Accuracy and Precision

2 μg/mL standard Ligustrazine Phosphate specimens was prepared in the same way as samples of standard curve, and processed according to the "sample processing", 6 aliquots were detected to obtain the accuracy.

2.6.3. Stability

Plasma and homogeneous liver samples containing 2 μg/mL standard 8 Ligustrazine Phosphate Liposome were prepared in the same way as samples of the standard curve, plasma samples were observed for 30 hours, the homogeneous liver samples were observed for 36 hours.

2.6.4. Calibration

Calibration specimens containing 0.5, 5, and 40μg·mL-1 standard Ligustrazine Phosphate were prepared in the same way as samples of the standard curve, and processed according to "sample processing". The variable coefficient should be no more than 15%.

2.7. Pharmacokinetic Calculation and Statistical Analysis

All samples were detected with HPLC-MS method. Pharmacokinetic parameters were calculated with DAS 2.1.1 software using non-compartmental analysis. Spss 11.5 software was involved to compare the concentration difference among all the 198 delivery systems.

3. Results

Ligustrazine Phosphate Liposome preparation and its quality control Ligustrazine phosphate liposome present itself as milky solution, the particle size of Liposome was approximately 200 to 300 nm. The centrifugal stability was perfect well, and it was lyophilized. The concentration was 33.3 mg/mL. At last, the EE was 87.98%.

3.1. Standard Curve and Quality Control

Eight concentrations were involved to calculate the standard curve. Concentrations of standard Ligustrazine phosphate was the horizontal axis, the peak area ratio of Ligustrazine phosphate under test to internal standard caffeine was the vertical axis, and linear regression was involved to estimate the linear regression equation, the weight coefficient was 1/C2. In plasma, the linear regression equation was y=2.145*X+0.980, R^2 =0.999; In liver tissue, the linear regression equation was y=2.383*X+0.005, R^2 212 =0.996. All samples were processed according to "Preparation of calibration standard in plasma and tissue", 50 μL blank plasma was processed for blank chromatogram, and 2μg·mL-1 Ligustrazine phosphate plasma solution was processed for Ligustrazine phosphate chromatogram and internal caffeine chromatogram. The relative retention time of ligustrazine phosphate was 12.3 min while caffeine was 9.3 min, so the ligustrazine phosphate and caffeine couldn't interfered with each other if detected under this chromatogram conditions, the speciality of this approach was prefect well. For lower limit of quantitation, six aliquots of 0.05μg·mL-1 ligustrazine phosphate was processed and detected, the RSD was 3.24%, and SNR was more than 10, which all hinted that the lower limit of quantitation was less than 0.05μg·mL-1. As recovery

rate, six aliquots of 0.6 μg/mL ligustrazine phosphate plasma solution was processed and detected for At in chromatogram, as a compare, the plasma was replaced with pure water, other procession were carried out as the same for A0 in chromatogram, the absolute recovery was 81.91±1.22%, in tissues, all absolute recovery was within 85±5%. For accuracy and precision, six aliquots of 2 μg/mL ligustrazine phosphate plasma solution were processed, the accuracy was. As stability test, 2μg/mL ligustrazine phosphate plasma and liver tissue sample were stored in room temperature for stability detection, the plasma sample could be stable for 30 hours while the tissue sample could be stable for at least 36 hours. At last, three concentrations of ligustrazine phosphate were prepared as quality control, and processed according to "sample processing", after detection, the absolute deviation was less than 15%, which proven that the detection result was reliable.

3.2. Acute Toxicity Test

The maximum administration level of Ligustrazine Phosphate Liposome was 100 mg/kg, equal to 567 mg per human, it was 11.34-fold of the clinical administration level in human. Pharmacokinetic Parameters of Single-Dose Ligustrazine Phosphate Lipidosome Intravenous injection of ligustrazine phosphate resulted in the direct appearance of substantial blood concentrations at the first time point of 5 min post dose both in 10 reference formulation and in Lipidosome formulation, and the effective concentration continued up to 240 min. However, the Lipidosome formulation released a higher smooth concentration since 240 min compared with the reference formulation. Examination of the blood concentration shed light on the long elimination half-life 246 time in lipidosome formulation (Figure 1). A summary of the pharmacokinetics parameters in blood were shown in (Table 1). AUC 0-∞ (mg/L*min) was 4271.975±913.101 for the reference formulation while 3635.768±745.612 for Lipidosome formulation, the Lipidosome formulation presented a long MRT 0-∞ (min) and t1/2 (min), which all revealed that the lipidosome formulation had a long surviving time during the treatment. What's meaningful, a large Vd/F (L/kg) in lipidosome formulation represented that the ligustrazine phosphate was widely distributed than in reference formulation. Despite all this, no significant difference of pharmacokinetics parameters in blood were existed between the reference formulation and lipidosome formulation.

Table 1. *Pharmacokinetic Parameters of Single-Dose Ligustrazine Phosphate Lipidosome.*

Pharmacokinetics parameters	The Reference Formulation	The Lipidosome Formulation
AUC 0-t (mg/L*min)	3956.665±858.604	3379.846±639.583
AUC 0-∞ (mg/L*min)	4271.975±913.101	3635.768±745.612
MRT 0-∞ (min)	63.731±12.886	66.481±22.438
t1/2 (min)	38.198±13.035	47.417±19.621
Tmax (mg/L)	14.167±9.174	6.667±4.082
Cmax (mg/L)	54.246±12.746	48.969±7.826
Cl/F (L/min/kg)	0.012±0.003	0.014±0.003
Vd/F (L/kg)	0.68±0.292	0.922±0.244

Note: Pharmacokinetics parameters were shown as Mean±SD.

3.3. Pharmacokinetics Parameters of Ligustrazine Phosphate Liposome in Tissues

Following a single injection of lipidosome formulation and reference formulation, ligustrazine phosphate appeared to be widely distributed in different tissues. The mean AUC 0-∞ (mg/L*min) in heart was 659.653±200.689 for lipidosome formulation while 586.67±46.635 for reference formulation, the difference was statistically significant, obviously, intake of ligustrazine phosphate in lipidosome formulation achieved more in heart. For liver, the mean Cmax was 12.084±4.368 in lipidosome formulation while 8.358±1.372 in reference formulation, so ligustrazine phosphate was absorbed more in liver, and it is the same as in lung. In kidney, the mean AUC 0-∞ (mg/L*min) was 957.048±157.049 for lipidosome formulation while 819.623±162.102 for reference formulation, the difference was statistically significant, which indicated that ligustrazine phosphate was distributed more in lipidosome formulation. As for plasma distribution, the Tmax of lipidosome formulation was longer than reference formulation, so ligustrazine phosphate was assimilated slowly in lipidosome formulation. What's important, the AUC (0-∞) and Cmax were 11 784.078±196.337 and 13.103±3.216 in lipidosome formulation (Table 2), respectively. Brain was distributed more when ligustrazine phosphate was in lipidosome formulation. Undisputedly, this act could contribute to the effective treatment.

Table 2. *Pharmacokinetics Parameters of Ligustrazine Phosphate Liposome in Tissue (Pharmacokinetics parameters were shown as Mean±SD).*

Pharmacokinetics	Heart		Liver		Spleen		Lung	
parameters	Reference	Lipidosome	Reference	Lipidosome	Reference	Lipidosome	Reference	Lipidosome
AU 0-t (mg/L*min)	557.873±45.72	532.164±51.495	757.976±255.249	799.043±81.431	471.86±85.903	471.027±49.704	415.915±49.833	407.973±43.053
AUC0-∞(mg/L*min)	586.67±46.635	659.653±200.689	795.687±249.719	906.46±103.387	545.4±117.988	530.263±62.02	438.373±69.666	444.9±23.743
MRT 0-∞ (min)	65.887±9.497	105.477±67.215	70.238±8.335	88.153±22.424	89.069±21.777	86.63±26.589	66.322±10.235	79.647±19.468
t1/2 (min)	41.604±13.02	71.943±47.874	42.766±9.448	59.06±16.246	56.528±15.778	55.975±21.771	38.887±9.761	51.785±20.809
Tmax (mg/L)	19.167±12.416	5±0	12.5±9.874	6.667±4.082	25±7.746	6.667±4.082	15.833±8.01	6.667±4.082
Cmax (mg/L)	6.972±0.63	8.743±3.76	8.358±1.372	12.084±4.368	5.931±1.007	6.715±2.401	5.702±0.836	7.135±2.663
Cl/F (L/min/kg)	0.086±0.007	0.081±0.02	0.069±0.023	0.056±0.006	0.095±0.021	0.096±0.012	0.116±0.018	0.113±0.006

Table 2. *Continued.*

Pharmacokinetics parameters	Kidney		Brain		Plasma	
	Reference	Lipidosome	Reference	Lipidosome	Reference	Lipidosome
AUC0-t (mg/L*min)	676.186±73.274	848.638±142.08	677.243±182.326	668.877±44.188	4393.688±443.78	3614.709±504.205
AUC0-∞(mg/L*min)	819.623±162.102	957.048±157.049	729.494±195.2	784.078±196.337	5618.374±770.43	109.709±25.912
MRT0-∞ (min)	97.232±34.816	85.479±27.652	77.574±14.512	89.23±42.495	113.486±23.538	109.709±25.912
t1/2 (min)	66.759±26.49	62.594±13.386	47.793±10.865	63.445±36.33	81.011±18.227	59.732±23.205
Tmax (mg/L)	18.333±9.832	9.167±10.206	32.5±42.866	5±0	10±5.477	35±12.247
Cmax (mg/L)	7.794±1.038	15.896±10.732	7.366±0.842	13.103±3.216	57.544±12.658	31.123±5.031
Cl/F (L/min/kg)	0.063±0.011	0.053±0.008	0.073±0.019	0.066±0.013	0.009±0.002	0.014±0.002

Note: ** represent P

4. Discussion

As a new drug delivery dosage formats and technologies, the liposomal formulation is considered to be a relatively nontoxic technology and can alter the distribution in targeting brain to improve the therapeutic index. Ligustrazine phosphate, as a drug widely used in China for the treatment of ischemic cardiovascular and cerebrovascular disease, the short elimination t1/2 and low bioavailability limited its treatment effect. Ligustrazine phosphate intravenous injection is distributed and eliminated rapidly in vivo. The phenomenon of short elimination t1/2 and low bioavailability is consistent with other reports. So we designed the liposomal formulation to improve the distribution in targeting brain. In our research, the liposomal formulation released a higher smooth concentration since 240 min compared with the reference formulation. Examination of the blood concentration shed light on the long elimination half-life time in liposomal formulation. The liposomal formulation presented a long MRT0-∞ (min) and t1/2 (min), which revealed that the liposomal formulation had a long surviving time during the treatment. What's meaningful, a large Vd/F (L/kg) n liposomal formulation represented that the ligustrazine phosphate was widely distributed than in reference formulation. Results in this study demonstrate that ligustrazine phosphate is rapidly absorbed and distributed following injection with a single dose of 50 mg/kg of body weight in rats. It can be seen that the tissue distribution after liposomal formulation injection was relatively high after ligustrazine phosphate injection in brain and heart. Compared with this ligustrazine phosphate injection, brain achieved a higher intake of ligustrazine phosphate in liposomal formulation, meaningfully, which meant that ligustrazine phosphate could exert its therapeutic action better in liposomal formulation via passive-targeting action. However, the mechanism of 12 biodistributionafter ligustrazine phosphate liposomal formulation administration needs to be clarified by further studies. In conclusion, the ligustrazine phosphate liposomal formulation could improve the ligustrazine phosphate bioavailability and brain targeting.

5. Conclusion

The results of the experiment show that the distribution of Ligustrazine Phosphate Injection liposome injection is relatively high in brain and heart tissue. Compared with Ligustrazine Phosphate Injection, the intake of tetramethylpyrazine phosphate in brain liposome preparations increased. It is known that tetramethylpyrazine phosphate liposome system can improve the bioavailability and brain targeting of tetramethylpyrazine.

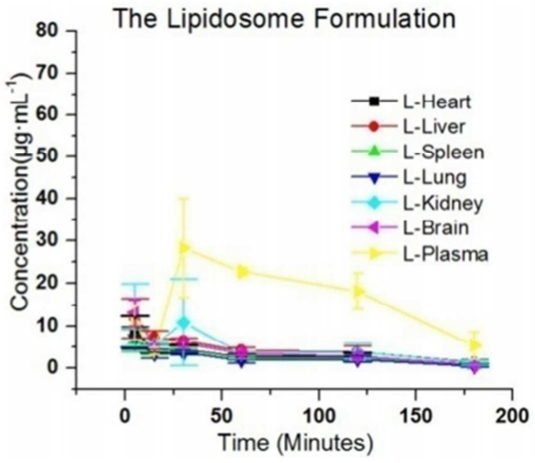

Figure 1. *Blood and tissues concentrations curve of Ligustrazine Phosphate.*

Abbreviations

LC/MS/MS: Liquid chromatograph/Mass spectrometer/ Mass spectrometer
DAS: Drug And Statistics
AUC: Area Under the Curve
TMP: Ligustrazine, 2,3,5,6-Tetramethylpyrazine
ESI: Eletro-spray Ionization
SIM: Selected Ion Monitoring
DLS: Dynamic Light Scattering
PDI: Polydispersity Index
EE: encapsulation efficiency
SNR: Signal to Noise Ratio
RSD: Rlative Sandard Deviation
MRT: Mean Retention Time

Acknowledgements

This work was funded by a grant from the Natural Science Foundation of Hebei Province in China (H2013201222), Hebei Administration of Traditional Chinese Medicine (No. 2013185, No. 2011106), and the National Natural Science

Foundation of Shan Xi (No. 2014021037-7). We also wish to thank Pu Jiang for translation assistance.

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