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Preparation and evaluation of Baicalin-loaded PLGA microspheres*in vitro* and *in vivo*

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Abstract

Here, the Baicalin-loaded PLGA microspheres (BC-MS) were prepared, and their properties *in vitro* and *in vivo* were evaluated. The microspheres were prepared using the solvent evaporation method based on O/W emulsion. The HPLC method was established in the determination of the content of baicalin in the microspheres. The surface and particle size were observed by the inverted microscopy, and the characteristics of *in vitro* release of BC-MS were investigated by dynamic dialysis method. After that, the microspheres were *in vivo* evaluated in rats. It was observed that the microspheres had an average particle size of 1.89μm, the drug loading was 12.79%, and the encapsulation rate was 85.40%.Moreover, the microspheres were spherical in shape and smooth surface with a uniform distribution. The release profiles of BC-MS agreed with Ritger-Peppas equation. The plasma concentration-time curves of BC-MS were fitted with two-compartment model. The results of pharmacodynamics in rats showed that: the elimination half-life of baicalin solution (BC-S) and Baicalin-loaded PLGA microspheres (BC-MS) were respectively 1.27 h and 258.98 h, mean residence time (MRT) were 0.88 h and 373.01 h, respectively. As the above result shows, BC-MS has the slow-release effect. Thus, the Baicalin-loaded PLGA microspheres have been successfully prepared.

Keywords: baicalin; PLGA microspheres; release profiles; pharmacodynamics

1. Introduction

Baicalin (BC, Figure 1), as a *Radix scutellariae* extract, is extracted from the dried roots of Scutellaria baicalensis Georgi (Labiatae). Baicalin has been found to have a variety of pharmacological effects, including anti-inflammatory [1,2], antioxidant [3], antiviral [4,5], antidiabetic [6], anticancer [7], and antitumor activities [8,9], cardio-protective effects [10-12] and so on. Clinically baicalinis used for infectious hepatitis, acute biliary tract infection, lead poisoning, upper respiratory tract infection,

hyperactivity, jaundice, dysentery, enteritis and keratitis [13]. Baicalin can be absorbed into the blood after enzymatic hydrolysis in the gut, and quickly transforms into scutellarein *in vivo*. However, the property of baicalin with poor solubility and stability, such as decomposition under alkaline conditions limits greatly its wide-spread application in clinical application [14]. As one of the widely accepted sustained release drug delivery systems, microspheres have been deeply investigated. It exerts sustained-release effects through the specific skeleton material, such as Poly(lactic-co-glycolic) acid (PLGA).PLGA is a biocompatible and biodegradable polymer [15], and which is decomposed into harmless products, H_2O and CO_2. PLGA was used as adjuvant which had been approved by the Food and Drug Administration (FDA), USA[16, 17].

Baicalin, as a hydrophobic drug, its internal absorption is affected by preparation factors greatly. The aim of the study is to prepare biodegradable baicalin-loaded microspheres with polymers. The objective of this paper is to reduce its stimulation and maintain drug concentration *in vivo* for a long time, so as to reduce the frequency of administration, decrease its side effects and improve patient compliance, thereby establishing the basis for further development of new form of baicalin.

2. Material and methods

2.1. Materials

Baicalin (purity 91%) was purchased from Aikang Pharmaceutical Co., Ltd. (Linyi, China). PLGA (ratio of lactic acid:glycolic acid of 75:25, average molecular weight 10,000Da) was provided from Daihang Biotechnology Co., Ltd.(Jinan, China). Gelatine, Dichloromethane and N, N-dimethylformamide (DMF) were obtained from Fengchuan Chemical Reagent Technology Co., Ltd. (Tianjin, China). Mannitol was provided from Aoboxing Biotechnology Co., Ltd. (Beijing, China). Dialysis bags (MWCO=7000Da, MD34) was purchased from Union Garbide (USA). All the reagents used were of analytical grade available from commercial sources.

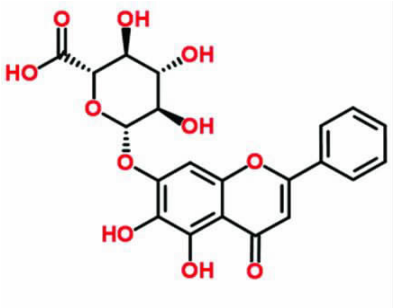

Figure 1. Structure of baicalin (BC)

2.2. Preparation of Baicalin-loaded PLGA-MS

2.2.1. Preparation of microspheres

The microspheres were prepared using the solvent evaporation method [18] based on O/W emulsion. In the process, baicalin and PLGA were dissolved in DMF and dichloromethane, respectively, and which was putted into three-necked bottle and used as the oil phase. Then the oil phase in the glass syringes with a No. 7 needle was dripped into the aqueous solution (pH=3) of gelatin to emulsify. Stirred at 1000rpm for certain time (2h) to volatilize solvent at water bath (20℃) using bainmarie (CSY-II, Beijing Medical Equipment Factory, China). Afterward, the samples were centrifuged at 3000 rpm for 10min using a mechanical stirrer (DSX-120,

Hangzhou Instrument Motor Co., Ltd, China.) and the supernatant was taken out. Then washed it three times with mannitol solution and then centrifuged at 3000 rpm for 5 min. The BC-MS was prepared after the process of lyophilization using freeze dryer (Eyela-FDU-1200, Tokyo Rikakikai Co. Ltd. Japan).

2.2.2. Experimental design

In this study, the orthogonal design [19,20] (Table 1) was introduced to optimize the formulation. In this design, PLGA concentration (A,W/V), dosage (B, mg), ratio of oil-water phase (C, V/V) and gelatin concentration (D, W/V) were selected for the factors that had significant effects on the properties of the microspheres based on the single-factor experiment previously. According to L9 (3^4)orthogonal arrays as shown in Table 1, the entrapment efficiency, drug loading and particle size were used as evaluation indexes, and weighted score method was used to optimize formulation on orthogonal design. The comprehensive score was described according to the following equation:

$$CS = EE(\%) \times 0.4 + DL(\%) \times 0.2 - AVS(\mu m) \times 0.4$$

where CS is the comprehensive score of the orthogonal design, *EE(%)* is the the entrapment efficiency of microspheres, *DL(%)* is the drug loading of microspheres, *AVS(μm)* the average particle size of BC-MS.

The levels and results of the orthogonal test factors were shown in tables 1, 2 and 3, respectively. Then three batches of BC-MS were prepared according to the optimal formula, and which were used to verify the test results (table 4).

Table 1. The orthogonal levels and factors of formulation

Level	Factors			
	A(W/V)	B(mg)	C(V/V)	D(W/V)
1	2%	40	1:5	1%
2	4%	60	1:10	1.5%
3	6%	80	1:20	2%

2.3. Characterization of microspheres

2.3.1. Microspheres content

The content of baicalin was analyzed by reversed phase HPLC using LC-20AT (Shimadzu international trade Shanghai ltd, China). The reversed phase C18 column of SHIM-PACK VP-ODS (150mm*4.6mm, 5μm) was applied in this study. The mobile phase was a mixture of methanol and pure water (50:50, v/v) containing 0.3% (v/v) orthophosphoric acid, constantly flowing at 1 ml·min^{-1}. The injection volume was 20μl and the detected wavelength was 277 nm. The drug loading, encapsulation efficiency and yield of the BC-MS were defined as the following formulation:

$$DL(\%) = \frac{W_{actual}}{W_{BC-MS}} \times 100$$

$$EE(\%) = \frac{M_{actual}}{M_{theoretical}} \times 100$$

$$Y(\%) = \frac{W_{BC-MS}}{W_{total}} \times 100$$

Where W_{actual} is the actual drug content in the weighed quantity of BC-MS, W_{BC-MS} is the weighed quantity of microspheres, $M_{theoretical}$ is the theoretical amount of BC in microspheres calculated from the initial quantity of BC added in prepared process. W_{total} is the total of drug and excipients in the process of the preparation.

2.3.2. *In vitro* release profiles

The characteristics of *in vitro* release of BC-MS were investigated by dynamic dialysis method. In the study, BC and BC-MS were accurately weighted 5mg into dialysis bag and dispersed in 5ml PBS (pH 7.4, containing 0.02% NaN_3) with shaking at a rate of 50rpm·min^{-1} in a dissolution vessel (ZRS-6G dissolution tester, Tianjin Tianda Tianfa Technology Co., Ltd., China)at 37.0℃. The dialysis bag was immersed in 200 ml PBS. The release medium(2ml) in the dissolution tester was collected and replaced with 2ml fresh release medium at the set time-points of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264h. The concentration of baicalin was measured using HPLC (LC-20AT, Shimadzu international trade Shanghai ltd, China), as described above. And the accumulative percentage was calculated using the following formulation:

$$Q\% = \frac{C_n \bullet V + V_i \sum_{i=0}^{n=i} C_i}{W \times DL\%} \times 100\%$$

where *Q%* represents the accumulative percentage, C_n is the concentration of the sample taken at the time-points of *n*, C_i is the concentration of the sample taken at the time-point of *i*, *V* is the total volume of the release medium, *W* is the total amount of microspheres, *DL%* is the drug loading of microspheres. Finally, the release curve was constructed [21].

2.3.3. Pharmacokinetics of BC-MS *in vivo*

In this part of the experiment, the pharmacokinetics of Baicalin-loaded PLGA microspheres (BC-MS) was investigated using SD(Sprague Dawley) rats (weight 220~250g, male, Tianjin Experimental Animal Center, China)via tail vein injection. In the study, 10 SD rats were randomly divided into the experimental group with BC-S and control group with BC-MS. Rats received BC-S together with saline and BC-MS (10.5 mg·kg^{-1}) via tail vein injection, respectively. And then blood was collected from the fundus venous plexus at the time-points of 5, 15, 30min and 1, 3, 4, 5, 6,7, 8, 9, 10, 11, 24, 36, 48, 96, 120h. The blood was placed in 5 ml plastic centrifuge tube wetted with heparin anticoagulation and centrifuged at the speed of 3000rpm for12min using centrifuge (LDZ5-2, Beijing Medical Centrifuge Factory, China). Then100μl of supernatant was taken and 200μl of methanol was added to deposit protein. Afterward, the samples were centrifuged at 4000rpm for20 min. Finally, the supernatant liquid obtained from the centrifugation was subjected to sample introduction and the concentration of baicalin was calculated.

2.3.4. The surface and particle size of BC-MS

The surface and particle size of BC-MS were observed by the inverted microscopy. Microspheres were suspended in distilled water and observed under the inverted microscopy (BMM-5500C, Shanghai Tong painted Optical Instrument Co., Ltd, China). The number of microspheres is not less than 200 in the field of camera(Toup View 3.7 For Digital Camera Imaging System, Hangzhou ToupTek Photonics Co., Ltd, China).

3. Results and Discussion

3.1. Preparation of microspheres

The microspheres were prepared by the solvent evaporation method based on O/W emulsion, using orthogonal design. It is mainly because of the hydrophobic nature of baicalin. Solvent evaporation method (solvent method), also known as liquid drying method, is a classic preparation of microspheres. The characteristics of microspheres showed the solvent evaporation was a suitable technique to prepare Baicalin-loaded PLGA microspheres (BC-MS).

Table 2. The design and results of the orthogonal experiment

No	A	B	C	D	Particle size(μm)	DL(%)	EE(100%)	Comprehensive score
1	1	1	1	1	9.23	18.21	38.24	15.25
2	1	2	2	2	13.38	31.47	60.32	25.07
3	1	3	3	3	11.40	9.69	16.71	4.06
4	2	1	2	3	14.77	17.73	42.11	14.48
5	2	2	3	1	9.78	22.35	72.27	29.46
6	2	3	1	2	7.30	26.66	73.99	32.01
7	3	1	3	2	5.29	12.55	83.46	33.78
8	3	2	1	3	7.29	14.91	70.32	28.19
9	3	3	2	1	10.13	18.31	69.11	27.25
K1	14.79	21.17	22.27	23.99				
K2	25.32	27.57	25.15	30.29				
K3	29.74	21.11	22.43	15.58				
R	14.95	6.47	2.89	14.71				

A: PLGA concentration (W/V); B: dosage (mg); C: ratio of oil-water phase (V/V);D: gelatin concentration (W/V); K1: average value of comprehensive score of level 1 of factors; K2: average value of comprehensive score of level 2 of factors; K3: average value of comprehensive score of level 3 of factors; R: range of the maximum and minimum.

3.2. The orthogonal design

Based on the Single-factor experiment previously, the optimal formula and preparation were obtained using analysis of range and variance of orthogonal experiment (Table 2). Range (R) indicates the effect of each factor on the test results. In the F test, The orthogonal design had 4 factors, A, B, C and D, and the orthogonal design did not take into account the experimental error, so took the factor of the minimum deviation square as error estimate to test the significant of other factors.

The results of orthogonal test showed that the influence of the four factors on the comprehensive score was A>D>B>C in Table 2. So, factor C was selected as the error estimate to test whether the other three factors had significant effect on the comprehensive score in the F test.

Table 3 showed the result that PLGA concentration (A) and gelatin concentration (D) had significant ($P<0.05$) influence on comprehensive score. The best combination was $A_3B_2C_2D_2$, the optimal preparation were as follows: 6% of PLGA concentration, 40mg of baicalin, 1:10 of oil and water ratio, 1.5% of gelatin concentration.

Three batches of BC-MS were prepared according to the optimal formula. The experimental results showed as follows: average particle size of 1.89μm, drug loading of 12.79%, entrapment efficiency of 85.40% and comprehensive score of 35.92 (Table 4).

Table 3. The results of F test

Factors	sum of square of deviations	df	F	F0.05(2,2)	Sig.
A	353.709	2	22.498	19.00	*
B	82.824	2	5.268	19.00	
C	15.722	2	1.000	19.00	
D	326.802	2	20.786	19.00	*
Error	15.72	2			

infuse : " * " , $P<0.05$

Table 4.The actual results of optimum preparation

Number	Particle size (μm)	DL(%)	EE(100%)	Comprehensive score
Y1	1.21	13.25	87.72	37.25
Y2	2.99	12.06	82.95	34.40
Y3	1.49	13.07	85.53	36.23

3.3. Characterization of microspheres

3.3.1. *In vitro* release

The most appropriate model was determined by comparing the correlation coefficient values (R^2) [22].The correlation coefficient values (R^2) was given out on the basis drug release data by data fitting method.

The *in vitro* release characteristics of baicalin from BC-MS and BC-S are shown in Figures 2 and 3. BC-S released 91.58% after 3h, however BC-MS released 32.20% in the first 24 hours. Then BC-MS released slowly and released 67.18% in 8 days, from this day, a burst release began with a higher release rate until the test was stopped. The study had demonstrated the sustained release of BC-MS over a 11-day period. The S-shaped curve showed that the rate of *in vitro* release of BC-MS was affected by the degradation of polymer carriers [23].

The results of model fitting *in vitro* release are shown in Table 5.The index n of time t is a characteristic parameter that characterizes the drug release mechanism. Comparing the correlation coefficient values (R^2), the release profiles of BC-S agreed with Weibull equation ($Q=100\times[1-e^{-((t+1.441)^{2.992})/41.163}]$, $R^2=0.9765$) and the release profiles of BC-MS had a good fit with Ritger-Peppas equation ($\ln Q=0.6525\ln t+1.5479$, $R^2=0.9920$). And then value of Ritger-Peppas equation was 0.6525. The study indicated that the release mechanism of BC-MS was non-Fick's diffusion and which was the result of a combination of the synergistic effect of diffusion and skeleton dissolution [24,25].

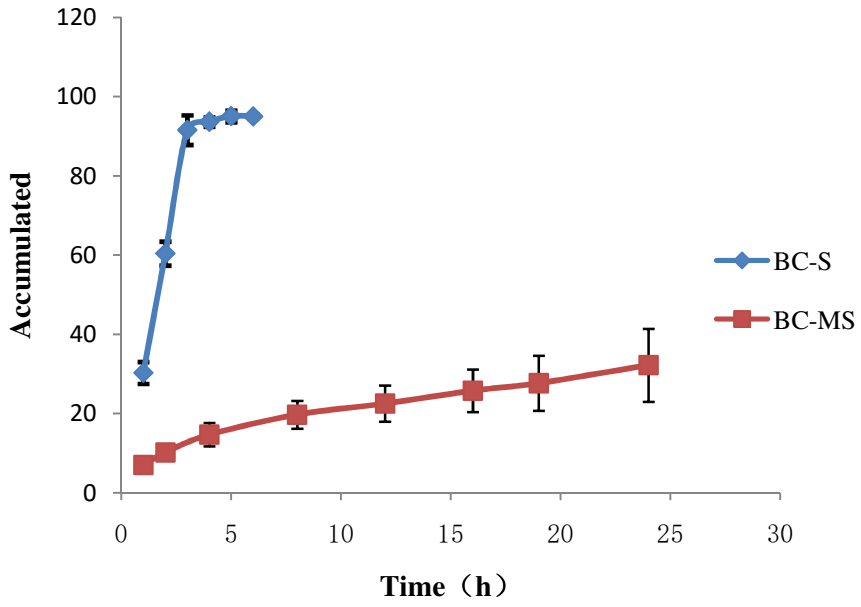

Figure 2. The release profiles of BC-S and BC-MS (0~24h) (n=3)

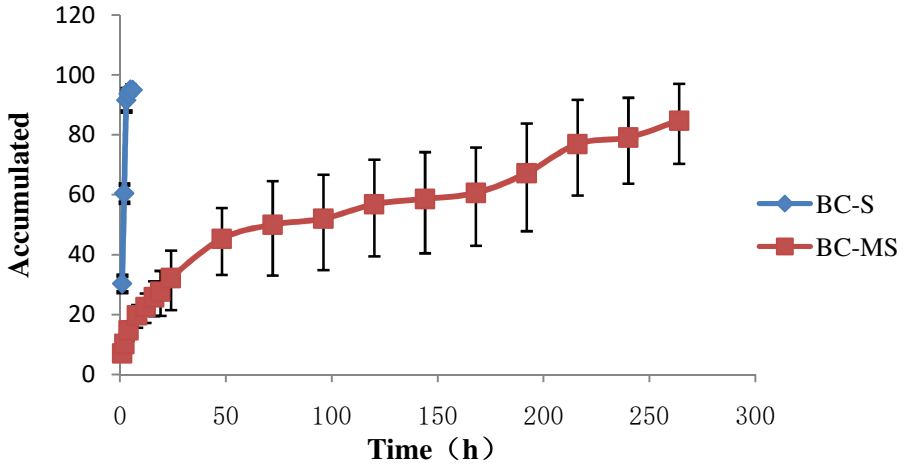

Figure 3. The release profiles of BC-S and BC-MS (0~264h) (n=3)

Table 5. The curve fitting equations of BC-S and BC-MS release *in vitro* against standard models

Mode	BC-S	BC-MS
Zero order	Q=22.131t+13.664	Q=0.26t+20.266
	(R^2=0.9100)	(R^2=0.9261)
First order	$Q=100\times[1-e^{-0.532t}]$	$Q=100\times[1-e^{-0.008t}]$
	(R^2=0.8819)	(R^2=0.8050)
Higuchi	$Q=43.193t^{0.5}$	$Q=5.229t^{0.5}$
	(R^2=0.8275)	(R^2=0.9261)
Weibull	$Q=100\times[1-e^{-((t+1.441)^{2.992})/41.163}]$	$Q=100\times[1-e^{-((t+1.568)^{0.594})/18.826}]$
	(R^2=0.9765)	(R^2=0.9787)
Ritger-Peppas	ln Q=0.441ln$t$+0.8975	ln Q=0.6525ln$t$+1.5479
	(R^2=0.8800)	(R^2=0.9920)

3.3.2. Pharmacokinetics of BC-MS *in vivo*

The Plasma concentration versus time curve (Figures 4 and 5) was obtained after intravenous administration of BC-S and BC-MS. The dates were analyzed by PKSolver software, and the analysis was based on the evaluation indicator of Akaike's Information Criterion (AIC). The pharmacokinetic parameters [26] in Table 7 were calculated according to the drug curve. The plasma concentration-time curves of BC-S and BC-MS were fitted with two-compartments model (Table 6). The results in Table 7 were that: the elimination half-life of BC-S

and BC-MS were 1.27 h and 258.98 h, respectively; the mean residence time (MRT) were 0.88h and 373.01h, respectively. The results showed BC-MS has more significant slow-release effect.

A kind of fluctuation phenomena appeared in the process of concentration of drug changing with time after intravenous administration of BC-MS, which may be due to the diffusion of drug and the degradation of polymer carriers. For example, the internal environment was complex, the physiological activity made the tissue fluid penetrate into the vicinity of the microspheres [27], and the exogenous antibodies that accumulated around the microspheres produced acid, free radicals and enzymes. It was because of these factors that PLGA of microspheres accelerated degradation.

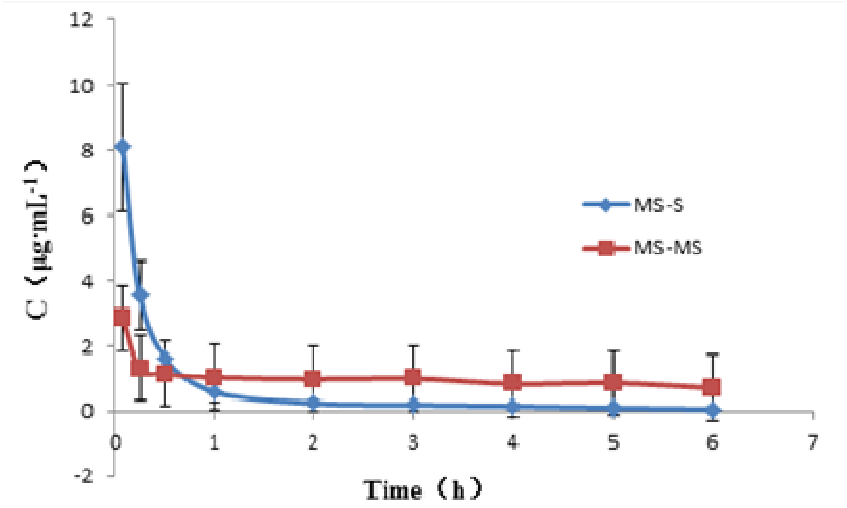

Figure 4. Plasma concentration-time profiles of BC-S and BC-MS (0~6h)(n=5)

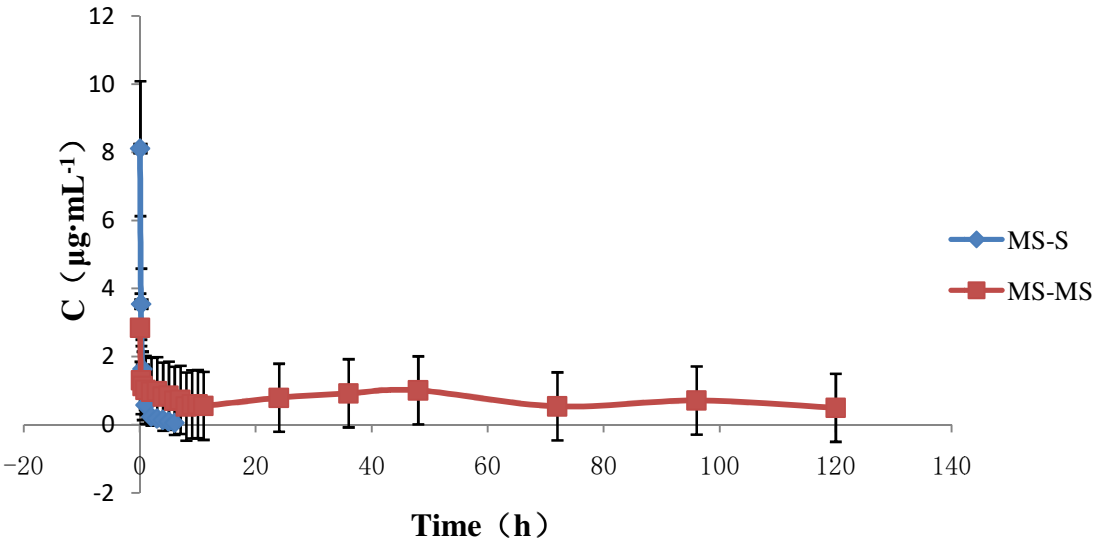

Figure 5. Plasma concentration-time profiles of BC-S and BC-MS(0~120h) (n=5)

Table 6. Akaike's Information Criterion (AIC) of BC-S and BC-MS applied with compartment model fitting

Formulation	AIC		
	One compartment	Two compartments	Three compartments
BC-S	-2.856	-0.95	6.18
BC-MS	33	-21.842	-12.44

Table 7. Pharmacokinetics parameters of BC-S and BS-MS after single intravenous injection in rats (n=5)

Parameter	Unit	BC-S	BC-MS
k10	1/h	4.21	0.01
k12	1/h	2.76	6.04
k21	1/h	1.66	1.34
t1/2α	h	0.12	0.09
t1/2β	h	1.27	258.98
CL	$mg \cdot mL \cdot h^{-1}$	2.93	0.03
AUC0-t	$\mu g \cdot h \cdot mL^{-1}$	3.65	85.01
AUC0-inf	$\mu g \cdot h \cdot mL^{-1}$	3.74	308.14
MRT	h	0.88	373.01

3.3.3. The surface and particle size of BC-MS

The inverted microscopy was used to identify the microspheres, and the surface and particle size met the requirements. The microspheres in product were light yellow powder of spherical in shape and smooth surface with a uniform distribution (Figures 6-8). The average particle size of BC-MS was 1.89μm (Figures 7-8).

4. Conclusion

Baicalin, as a hydrophobic drug, its internal absorption is affected by preparation factors greatly. In this study, the Baicalin-loaded PLGA microspheres (BC-MS) were prepared using the solvent evaporation method based on O/W emulsion. The orthogonal experimental design was introduced to optimize the formulation. The microspheres in product were light yellow powder of spherical in

shape and smooth surface with a uniform distribution. The results showed that the microspheres increased the release rate of the drug, and had more significant slow-release effect. Thus the use of BC-MS would be beneficial for patients. Therefore the study establishes the basis for further development of new form of baicalin.

Figure 6. The appearance of the baicalin-loaded PLGA microspheres

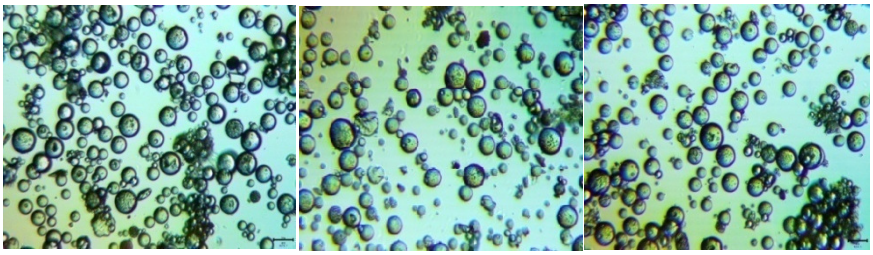

Figure 7. Microspheres of the baicalin-loaded PLGA microspheres(×400)

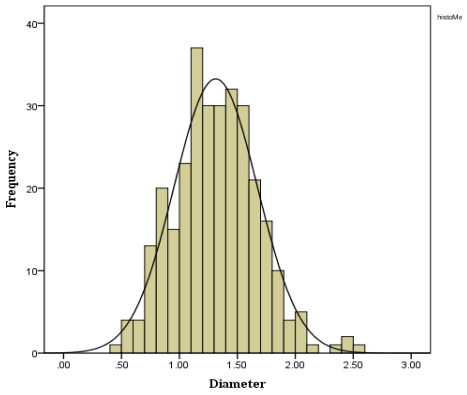

Figure 8. The particle diameter distribution of optimum prescription

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