ORIGINAL ARTICLE

Preparation and characterization of Biochanin A loaded solid lipid nanoparticles

Chunlei Tao[1,2,3], Hailin Cheng[4], Kai Zhou[1], Qing Luo[1], Lili Guo[1], Weidong Chen[1,2,3]

[1]Department of Clinical Pharmacy in College of Pharmacy, Anhui University of Traditional Chinese Medicine, Hefei, Anhui 230031, [2]Anhui Engineering Research Center for Modern Chinese Materia Medica, Anhui Hefei 230031, [3]Anhui Key Laboratory for Modern Chinese Materia Medica, Anhui Hefei 230031, [4]Anhui Feidong Province People's Hospital, Anhui Hefei 231600, China

Biochanin A, the predominant isoflavones found in plants, had proved its human health benefits. The purpose of this research was to study whether Biochanin A (BCA) loaded solid lipid nanoparticles (SLN) could improve solution and prolong the half-life of BCA. BCA-SLN was prepared by emulsion evaporation and low temperature solidification technique, and freeze-dried powders were developed to improve stability. The mean particle sizes, zeta potential, entrapment efficiency (EE), and drug loading capacity (DL) of BCA was 176.0 nm, −18.7 ± 0.26, 97.15 ± 0.28%, and 6.38 ± 0.04%, respectively. The results of differential scanning calorimetry (DSC) and X-ray diffraction analysis (XRD) indicated that the BCA was wrapped and absorbed in the nanoparticles. The solution of preparation is much higher than the untreated BCA. Results of stability of SLN showed a relatively short-term stability after storage at 4°C and 25°C for 15 days. Drug release of untreated BCA and BCA-SLN was fit into the Biexponential equations and Weibull equations, respectively, and SLN showed sustained release properties. But after freeze-dried, stability was improved, and the EE and DL had a slightly decrease. The mean particle size was slightly increased, but the structure was not changed. In conclusion, SLN systems can represent an effective strategy to change the poor aqueous solubility and prolong the half-time of BCA.

Key words: *Biochanin A, stability, freeze-dried powders, enhanced dissolution, solid lipid nanoparticles*

INTRODUCTION

Phytoestrogens are plant-derived compounds that structurally or functionally mimic mammalian estrogens. Isoflavones are the most important type of phytoestrogen found in legume plant,[1] and are week estrogenist receptor ligands with mixed agonist-antagonist activity.[2] Biochanin A (BCA,5,7-dihydroxy-4′-methoxyisoflavone), one of the predominant isoflavones, existing in red clover, cabbage, alfalfa and Trifolium lucanicun Gasp,[2,3] has been associated with a variety of human health benefits. Firstly, due to the similar structure to estrogens, BCA can combined with estrogen receptor α (ERα) and β (ERβ) which be called estrogenic activity.[4] Secondly, BCA has various other biological activities, such as anti-proliferative, anti-inflammatory,[5] protection of dopaminergic neurons,[1] stimulation of osteoblastic differentiation,[6] and inhibition of melanogenesis.[7] Especially to deserve to be mentioned, data from the animals and the *in vitro* studies provided that Biochanin A, which are possibly through the path of inhabiting the enzyme activity and inducting apoptosis, can reverse, inhabit, or prevent cancers or tumor development, such as prostate cancer,[8] breast cancer,[9-11] lung tumor,[12] liver cancer[13] and others.[14,15]

The anticancer ability of Biochanin A is also own to the role as an inhibitor of P-glycoprotein, a major efflux transporter protein,[16] which has a great impact on the absorption, distribution and elimination of various therapeutic compounds.[17] But the *in vivo* studies indicated that BCA had failed to improve the bioavailability of P-gp substrate by oral administration when comparing with the *in vitro* results.[18] A precious study indicated BCA has high clearance, a large apparent volume of distribution and a poor oral bioavailability (only 2.6% after the 5 mg/kg dose and 1.2%

Address for correspondence:
Dr. Weidong Chen,
45 Shihe Road, Anhui University of Traditional Chinese Medicine,
Hefei, Anhui, China.
E-mail: anzhongdong@126.com

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after the 50 mg/kg dose after oral administered) in rats.[19] So The discrepancy between *in vitro* and *in vivo* results may cause by the low solubility, thus low oral bioavailability of BCA itself. Recently, BCA via the preparation of solid dispersion has been employed to address the issues related to poor aqueous solubility and low bioavailability of BCA.[18] Furthermore, pharmacokinetics studies in rats indicated that solid dispersion formulation significantly improved the oral exposure bioavailability of BCA.[18] There studies provided a hint about improving the oral bioavailability of BCA via other formulations.

With the development of science and technology and the rising level of preparation, more and more attention have been taken to traditional colloidal drug carriers, such emulsions, liposomes, and polymeric micro- and nanoparticles, which have been demonstrated as a promising technique for improving the solubility and dissolution rate of poorly water soluble drugs.[20,21] Among them, solid lipid nanoparticles (SLN), a lipid-based formulation, appeared to be highly effective to enhance the oral bioavailability of some of the most poorly absorbed compounds, and take the advantage of polymeric nanoparticles, fat emulsions and liposomes.[22,23] Recently, more and more studies reported the possible mechanisms of SLN for improving the oral bioavailability of poorly soluble drugs. For example, the ability of SLN as lipid vehicles could help drugs target into lymphatic delivery system,[24] which is helpful to the absorption of poorly soluble drugs by avoiding the first-pass metabolism.[25] Actually, not only the structure of SLN, but the surfactant added in the SLN, such as Tween 80, could also contribute to the improved bioavailability of SLN.[26] These advantages of SLN can be provided to various drugs, no matter hydrophilic or lipophilic drugs.[27]

The aim of this work was to conquer the low solubility of BCA by preparing BCA-loaded solid lipid nanoparticles (BCA-SLN), and investigate the physicochemical characteristics and drug release *in vitro*. All the parameters of BCA-SLN were compared with the free BCA.

MATERIALS AND METHODS

Chemicals and reagents

BCA (>99.0% purity grade, number is CY110121) was purchased from Shanxi Ci Yuan Bio-technology CO., Ltd (Shanxi, China). Poloxamer 188 (Pluronic F 68) was obtained from Beijing Fengli Jingqiu Commerce and Trade Co., Ltd (Beijing, China), and Tween-80 was from National Pharmaceutical Group Chemical Reagent Co., LTD (Shanghai, China). Lecithin was generously supplied by Anhui BBCA Pharmaceutical Co., Ltd (Hefei, China). Glycerol monostearate (GMS) was donated by Anhui Sheng Ying Pharmaceutical Co., Ltd (Hefei, China). Methanol and acetonitrile were HPLC grade. All other chemicals and solvents were of analytical reagent grade and were used without further purification. The water used for all experiments was distilled water.

Preparation of solid lipid nanoparticles and lyophilization

The BCA-SLN was prepared by emulsion-evaporation and low temperature-solidification technique.[28] The lipid and aqueous phases were prepared separately. Briefly, GMS (100 mg), Lecithin (100 mg) and BCA (7.5 mg) were mixed and dissolved into 5 ml solvent (ethanol:Acetone = 2:1) in a water bath at 70°C to obtain the organic phase. At the same time, 10 ml aqueous phase (Tween-80/Pluronic F 68 of 1:1 as emulsifier mixture, both 180 mg) had been prepared and heated to the same temperature of the organic phase. To obtain the coarse emulsion, the hot organic solution was injected into the aqueous phase under rapid stirring at 1000 rpm for dispersion. After that, the pre-emulsion was rapidly poured into ice bath (0-2°C) and stirred with magnetic stirring apparatus for 1 h.

BCA-SLN was formed during the period of solvent evaporation. The formulation was stored at 4°C. Then the solution of formulation was freeze-dried in −86°C Ultra low temperature freezer (Zhongke Meiling low temperature science and technology Co., Ltd, Hefei, China) for 36 h after adding lyoprotectant (7% of mannitol) and pre-freezing in −4°C freezer (Meiling Group Co., Ltd, Hefei, China) for 12 h.

Characterization of solid lipid nanoparticles

Particle size and zeta potential

The SLN was characterized for their size, PDI and zeta potential with a Malvern Zetasizer NanoZS90 (Malvern Instruments Ltd., Malvern, UK). Zeta potential measurements were done at 25°C. All the samples were diluted to an appropriate concentration by distilled water before the measurement.

Drug loading and entrapment efficiency

The ultrafiltration technique was to use to measure the concentration of free drug in the dispersion medium in order to determine the drug loading (DL) and entrapment efficiency (EE).[26] 5 ml of sample of BCA-SLN was added into Amicon Ultra-4 ultrafiltration device (molecular weight cut-off was 100 KDa, Millipore) and centrifuged (LC-4016, Anhui USTC zonkia scientific instruments Co., Ltd, China) at 3500 rpm for 30 min. Then the filtrates were filtered with 0.45 μm filter membrane and determined by HPLC method. The assay was repeated three times using different samples from independent preparations. DL and EE are calculated by the following equations:

$$\%DL = (W_{initial\ drug} - W_{free\ drug})/W_{lipid} \times 100\% \quad (1)$$

$$\%EE = (W_{initial\ drug} - W_{free\ drug})/W_{initial\ drug} \times 100\% \quad (2)$$

Where the $W_{initial\ drug}$ is the weight of drug added in the system; $W_{free\ drug}$ is the weight of drug in the filtrate and the W_{lipid} is the weight of total lipid added in the system.

Transmission electron microscopy of nanoparticles

The morphology of nanoparticles was performed by TEM (JEM-1200EX, JEOL Co., Ltd, Japan) using a negative-staining method. A drop of BCA-SLN dispersions and freeze dried powders of BCA was spread on a copper grid, respectively, and dried at room temperature for about 20 min. Then the samples were gained for the TEM investigation.

X-ray diffraction analysis

X-ray diffraction patterns (XRD) are widely used to evaluate the physical nature and inner structures of the lipid nanoparticles.[29] In this study, X-ray diffractometer (Beijing Persee General Instrument Co., Ltd, China) was used to investigate the changes in the crystal structure. Diffractograms were performed from the initial angle $2\theta = 2°$ to the final angle $2\theta = 50°$ at a scanning rate of 4°/min, and the electric field strength was 36 kV and 25 mA.

Differential scanning calorimetry analysis

Differential scanning calorimetry (DSC) analysis was performed on a Q2000 DSC detector (American TA Instrument Co., Ltd, Delaware). Approximately 1-10 mg of sample was weighed into an aluminium pan and sealed hermetically, and the thermal behavior determined in the range of 25 to 250°C at a heating rate of 10°C/min under a nitrogen purge, using an empty pan as reference. The samples used for these analysis were (1) BCA; (2) Lecithin; (3) F68; (4) GMS; (5) physical mixtures (BCA, lecithin, F68 and GMS); (6) lyophilized SLN with loaded BCA (without lyophilization); (7) Mannitol; (8) lyophilized SLN with loaded BCA (lyophilization).

Stability test

The changes in the particle size and EE of SLN were widely used as indicators of storage stability.[30,31] Three batches of BCA-SLN and freeze-dried powders were stored at 4°C and 25°C for 1 month. The physical stability of the samples were evaluated on 0 days, 15th, 20th and 30th for any change in particle size, zeta potential and EE. The same detection was evaluated for freeze-dried powders of BCA-SLN on 0 day, 15th and 30th.

In vitro drug release studies

The drug release was studied by dialysis bag diffusion technique.[32] The dialysis bag (molecular weight cut off between 8000 and 14000) was soaked in double-distilled water for 12 h before using. BCA-SLN (equivalent to 1 mg of BCA) solution was poured into dialysis bag with the two ends fixed by thread and completely immersed in 250 ml of pH 7.4 phosphate buffer solution (PBS). The suspension was stirred at 37 ± 0.5°C and the stirring speed was set at 75 rpm. The samples (2 ml) were removed from the receiver solution, and an equivalent amount of fresh medium was added to keep a constant amount at predetermined time points (10 min, 15 min, 30 min, 45 min, 60 min, 90 min, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 36 h, 48 h). Free BCA suspended in dissolution medium was used as a control. The samples were filtered with 0.45 μm filter membrane, and 20 μl of continued filtrate were injected into HPLC system to detect the concentration of BCA.

HPLC assay for BCA

Shimadzu LC-15C PHLLC system equipped with LC-Solution lite Chinese chromatography data system (Shimadzu Co., Ltd., Japan) was used for quantitative determinations. HPLC analyses were performed on a COSMOSIL C_{18} column (4.6 mm × 250 mm, 5 μm, Nacalai Lnc, Japan) at 30°C. The mobile phase was contained acetonitrile/0.1% phosphoric acid (60:40, v/v %) and pumped at a flow rate of 1.0 ml/min. The injection volume was 20 μl, and the detective wavelength was set at 260 nm. The retention time of BCA was 6.8 min. Linear calibration curve was obtained for BCA in the range of 0.0050 ~ 5.0 μg/ml with r = 1.0000. While linear calibration curve *in vitro* was obtained for BCA in the range of 0.0050 ~ 1 μg/ml with r = 0.9999.

Statistical analyses

All statistical analyses were performed using statistical package for social sciences (SPSS, version 17.0). The data were presented as mean ± SD, and the Student's *t* test was

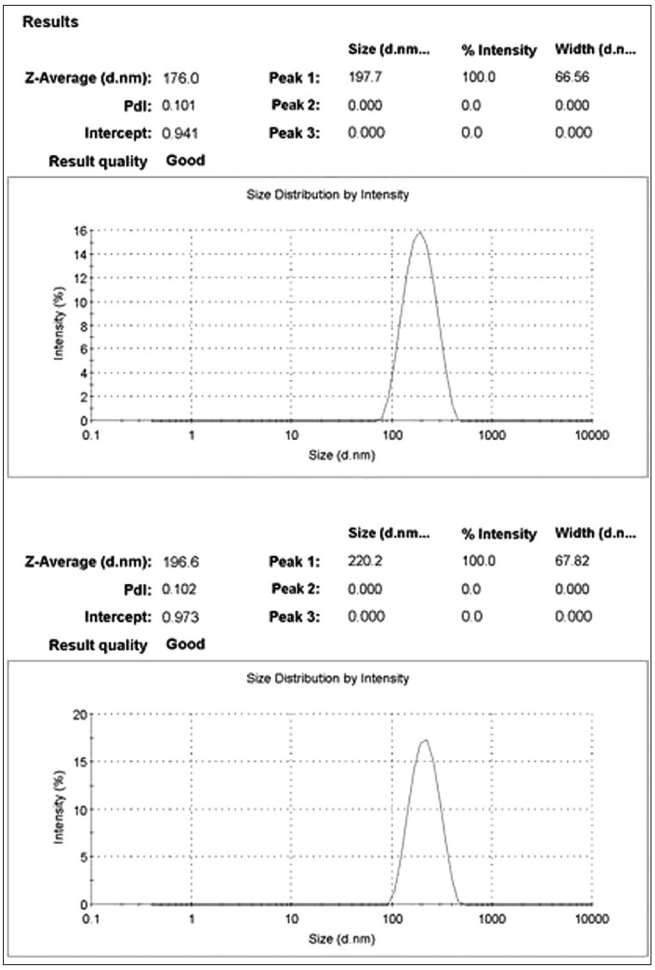

Figure 1: The particle size distribution of the BCA-SLN and freeze-dried powders of BCA-SLN (A: The size distribution of BCA-SLN; B: The size distribution of freeze-dried powders of BCA-SLN)

used to analyze differences between the two groups. A value of $P \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Characterization of SLN

Particle size and zeta potential

The values of the particle size and zeta potential of BCA-SLN and freeze-dried powders of BCA-SLN were documented in Figure 1 and Table 1.

The mean diameter of BCA-SLN was 176.0 nm, while the mean diameter of freeze-dried powders of BCA-SLN was 196.6 nm. The mean zeta potential of BCA-SLN was -18.7 ± 0.26mV ($n = 3$), whereas the mean potential of freeze-dried powders of BCA-SLN was -18.9 ± 1.05 mV ($n = 3$). The typical particle and zeta potential distribution of the BCA-SLN and freeze-dried powders of BCA-SLN were shown in Figure 1. On the basis of the data we got that the particle size was in narrow distribution and the lyophilization process had a little effect on parameters. But the zeta potential was a little low in some reports.[20,33]

Entrapment efficiency and drug loading

The EE and DL were two important indicators about the preparation evaluation of the BCA-SLN and freeze-dried powders of BCA-SLN. The EE and DL of SLN were $97.15 \pm 0.28\%$ (RSD = 0.14%) and $6.38 \pm 0.04\%$ (RSD = 1.91%), and EE and DL of freeze-dried powders were $96.68 \pm 0.10\%$ (RSD = 0.15%) and $6.24 \pm 0.03\%$ (RSD = 0.28%). As the result, the BCA loaded in SLN has higher EE and DL. And EE and DL of BCA-SLN had a little changed after freeze-dried.

TEM investigations

The TEM image of the BCA-SLN and freeze-dried powders of BCA-SLN were shown in Figure 2. The photographs of TEM about BCA-SLN and freeze-dried powders showed that the nanoparticles were both in shape of spherical well dispersed with approximately homogeneous in sizes and almost no adhesive.

X-ray diffraction analysis

In order to investigate the changes of the microstructure in the lipid crystallization process, X-ray diffraction experiments were performed.[34] The XRD patterns of the untreated BCA, F68, GMS, Lecithin, their physical mixture (PM) and lyophilized BCA-SLN (without lyphilization) were showed in Figure 3. There are more than 10 characteristic peak in the figure of BCA (from 5° to 30°), and these patterns indicate crystalline nature. Compared to the untreated BCA, the peak intensities of PM were decreased, which showed that the degree of crystallinity of BCA reduced. In contrast, the intensity and location of peaks were changed obviously in BCA-SLN, which indicated that solid lipid nanoparticles had crystal defects and less ordered structure.

Table 1: The zeta-potential of BCA-SLN and freeze-dried powders of BCA-SLN

Samples	BCA-SLN			Freeze-dried powders of BCA-SLN		
	1	2	3	1	2	3
Zeta potential (mV)	−18.9	−18.8	−18.4	−17.8	−18.9	−19.9
Mean±SD	−18.7±0.26			−18.9±1.05		

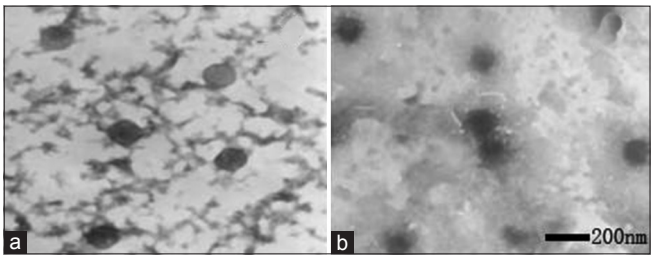

Figure 2: Photographs of BCA-SLN and freeze-dried powders of BCA-SLN: (a) BCA-SLN (magnification ×10000); (b) freeze-dried powders of BCA-SLN (magnification ×12000)

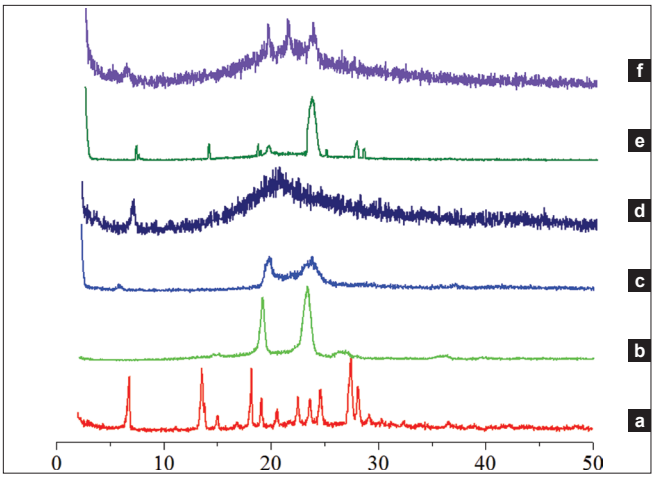

Figure 3: X-ray diffraction patterns of BCA-SLN (a) BCA; (b) F68; (c) GMS; (d) Lecithin; (e) PM; (f) BCA-SLN (without lyoprotectant)

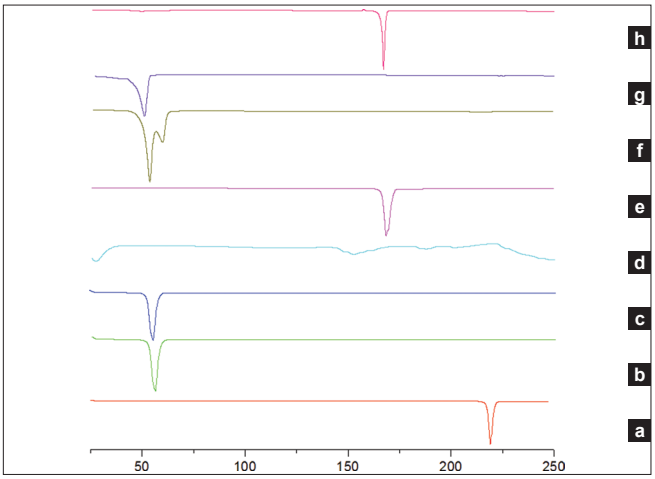

Figure 4: DSC curves (a) BCA, (b) F68, (c) GMS, (d) Lecithin, (e) Mannitol, (f) PM, (g) lyophilized BCA-SLN (without Mannitol), (h) lyophilized BCA-SLN (with Mannitol)

We can conclude that BCA-SLN has a new kind of structure which is much different from the PM. It might be caused by the fact that BCA was encapsulated into or absorbed to the surface of nanoparticles.

DSC analysis

DSC is frequently used to measure the heat loss or gain resulting from physical or chemical changes within a sample as a function of the temperature.[35] And it gives an insight into the melting and recrystallization behavior of crystalline material like lipid nanoparticles.[36] Figure 4 shows DSC curves of BCA, F68, GMA, Lecithin, Mannitol, PM, lyophilized BCA-SLN (without Mannitol) and lyophilized BCA-SLN (with Mannitol). The thermogram of the lyophilized BCA-SLN (without Mannitol) and BCA-SLN (with Mannitol) did not show the melting peak for BCA around 219°C. This showed that BCA was not in crystalline state. However, it was confirmed by the presence of melting peak of BCA in the PM. The causes of the phenomenon may because that BCA was coated or absorbed by SLN. Endothermic peak of Mannitol used as cryoprotectant was observed at 166.7°C in lyophilized BCA-SLN (with Mannitol) curve,[31] lyophilized BCA-SLN (without Mannitol) and BCA-SLN (with Mannitol). There is a melting peak around 50°C, but which was not existed in PM. And these implied that a new structure was formed in BCA-SLN, which was consistent with the XRD analysis. And compared to the melting-peak in BCA-SLN (with or without Mannitol), the behavior of lyophilized had no effect on the structure of SLN.

Stability test

The value of the mean particle size and EE ($n = 3$) were shown in Figures 5 and 6. Among the initial 15 days, the BCA-SLN solution was stable at 4°C and 25°C. But after 20 days, there were drug exudations both in 4°C and 25°C storage conditions. However, after freeze-dried processing, it could be very stable in 30 days. And in the figure, the column chart expressed the EE% of BCA-SLN, and line chart expressed the mean particle sizes of BCA-SLN.

In vitro release study

Dissolution mediums with different pH values may result in different solubility. We selected five different dissolution mediums to dissolve the BCA-SLN. As shown in Figure 7, the solution improved as the PH increased. Therefore, we selected pH 7.4 PBS as the dissolution medium. The solution of freeze-dried powders of BCA-SLN in water is 444.72 ± 0.46 μg/ml (n = 3), which is much higher than the solution of untreated BCA in the same medium (approximately 6.73 ± 0.36 μg/ml). In this study, the dialysis bag diffusion technique was chosen to investigate the release from BCA-SLN in pH 7.4 PBS (37 ± 0.5°C) (n = 3).

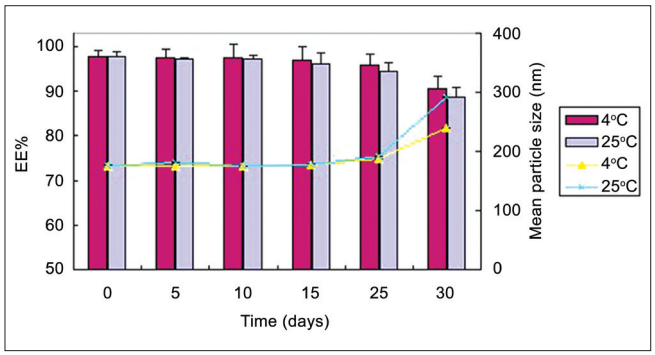

Figure 5: Mean particle size and EE% of BCA-SLN after storage at various stress conditions (please note different scale of secondary y-axis)

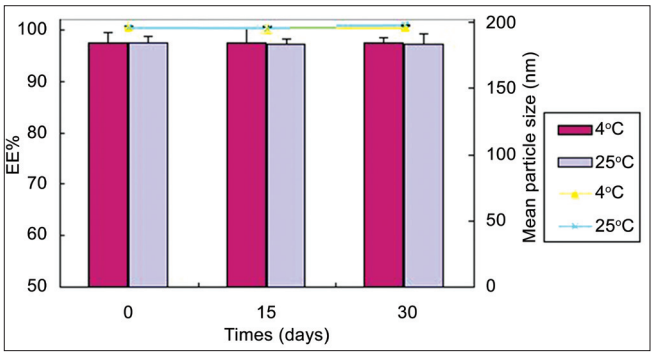

Figure 6: Mean particle size and EE% of freeze-dried powders of BCA-SLN after storage at various stress conditions (please note different scale of secondary y-axis)

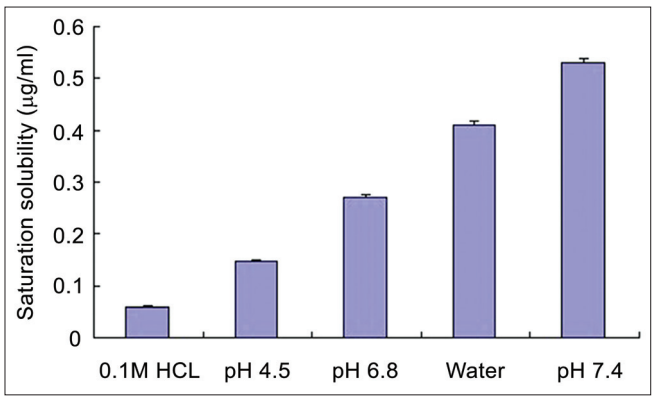

Figure 7: The results of saturation solubility about BCA

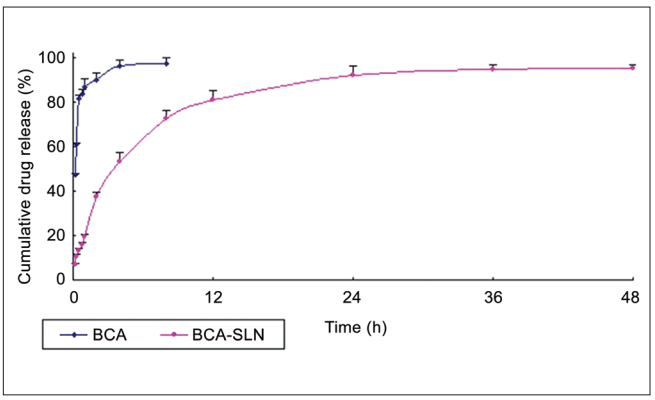

Figure 8: The release profile of BCA from BCA-SLN and BCA

Table 2: The regression equation of BCA released from BCA-SLN *in vitro*

Drug or preparation	Model	Equation	Correlation coefficient (r)
BCA	Zero order kinetics	Q=7.889t +52.688	0.5794
	First order kinetics	ln(100-Q)= −0.466t+3.642	0.8422
	Higuchi	$Q=50.916t^{1/2}+21.626$	0.8422
	Weibull	ln [-ln(1-Q%)]=1.657lnt+0.570	0.8985
	Nibbergull	$(1-Q\%)^{1/2}= -0.0363\ t+0.955$	0.9721
	Biexponential	$1-Q\%=0.948e^{-3.138\ t}+0.161\ e^{-0.247\ t}$	$r_\alpha=0.9892$, $r_\beta=0.9319$
BCA-SLN	Zero order kinetics	Q=1.989t +25.329	0.8193
	First order kinetics	ln(100-Q)= −0.07 t+4.309	0.9499
	Higuchi	$Q=0.0571^{1/2}-0.285$	0.9346
	Weibull	ln [-ln(1-Q%)]=1.430 lnt-1.392	0.9953
	Nibbergull	$(1-Q\%)^{1/2}=-0.0173\ t+0.858$	0.8422
	Biexponential	$1-Q\%=0.961e^{-0.186\ t}+0.370\ e^{-0.0488\ t}$	$r_\alpha=0.9940$, $r_\beta=0.9514$

As shown in Figure 8, approximately 97% BCA was released within 8h. While when approximately 95% BCA was released from BCA-SLN, the time it lapsed was prolonged to 48 h. These indicated that SLN possessed a sustained release. The release pattern of drug was analyzed by Zero order kinetics, First order kinetics, Higuchi, Weibull, Nibbergull and Biexponential equations. These release patterns of BCA were found to follow Biexponential equations ($1-Q\% = 0.948e^{-3.138t} + 0.161e^{-0.247t}$, $r_\alpha = 0.9892$, $r_\beta = 0.9319$), while release patterns of BCA-SLN were found to follow Weibull eqution ($\ln[-\ln(1-Q\%)] = 1.430\ln t-1.392$, $r = 0.9953$). The regression equation was shown in Table 2.

CONCLUSIONS

The BCA loaded solid lipid nanoparticles was prepared by emulsion-evaporation and low temperature-solidification technique, and effectively enhanced the dissolution of BCA. Besides, the freeze-dried method was succeeded to prepare the freeze-dried powders, and the stability of BCA-SLN had been improved. In this study, the result of *in vitro* experiment indicated that BCA loaded SLN can possess a sustained release. Therefore, SLN systems can represent an effective strategy to change the poor aqueous solubility and prolong the half-life of BCA.

So far, we still need some pharmacokinetics results to directly prove that SLN formulation could help to overcome the oral bioavailability problems of BCA. So we plan to finish the *in vivo* experiment in next step. The nanostructured lipid carrier's formulation (NLC) of BCA has also in our consideration.

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Authors Chunlei Tao and Hailin Chen contributed equally to this work. We also thank Author Qiang Wang for his assistance in this experiment.

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