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Preparation and evaluation of the curcumin niosomes: *In vitro* and *in vivo*

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Abstract: The curcumin niosomes were prepared and evaluated. The film dispersion method was applied, the formulation was optimized by single factor experiment and central composite design. The optimum formulation was as follows: the ratio of Span80 to cholesterol was 4.2:1, the ratio of cholesterol to curcumin was 5:1, PBS volume was 20.9 mL, hydration speed was 381 r/min, hydration time was 1.5 h, temperature was 50°C. The encapsulation efficiency of curcumin niosomes was 88.5%, average particle size was 162 nm, the Zeta potential was (-28.9±2.7) mV and the shape was regular. *In intro*, the niosomes exhibited good delayed release characteristics, and the drug release was in accordance with Ritger-peppas model. *In vivo*, the mean retention time ($MRT_{(0-t)}$) of curcumin niosomes (6.604±0.209 h) was significantly extended than that of the curcumin suspensions (2.498±0.016 h); the $AUC_{(0-t)}$ of niosomes (2074.989±146.690 ng·mL^{-1}·h) was significantly larger than that of the suspensions (803.475±23.335 ng·mL^{-1}·h), the relative bioavailability was 258.25%. The study showed a great potential of curcumin niosomes as a good formulation with improved oral absorption.

Keywords: Curcumin niosomes, delayed release, pharmacokinetics.

INTRODUCTION

Niosomes were prepared with cholesterol and nonionic surfactants, which had a stable double-layer structure. According to reports, niosomes have many advantages such as good biocompatibility, low toxicity, no immunogenicity, strong solubilization ability, stable drug loading, wide route of administration, sustained release and targeting effect (Zhang *et al*, 2015). Compared with liposomes, the application prospect of niosomes is broader because niosomes are more stable and not easy to be oxidized.

As a hydrophobic polyphenol, curcumin possess important biological and pharmacological activities such as anti-inflammatory (Ullah *et al*, 2017), antioxidative (Momeni and Eskandari, 2017), anticoagulant (Li *et al*, 2016), antibacterial (Liu *et al*, 2016), anticancer (Subramani *et al*, 2017), antiatherosclerosis (Zheng *et al*, 2016), antihypertensive (Heni *et al*, 2016), anti ulcer (Wu *et al*, 2020), antiviral (Mounce *et al*, 2017), lipid-lowering (Qin *et al*, 2015) and anti-aging (Yang *et al*, 2017) activities. Because of the pharmacological safety and efficacy, curcumin becomes a potential compound for treatment and prevention of cancer and other diseases. However, the poor relative bioavailability of curcumin caused by its extremely low solubility and stability was a major problem for its application.

At present, various strategies may be formulated to improve the bioavailability of curcumin, such as chelation strategies (Lin *et al*, 2017), hydrogels (Zhou *et al*, 2019), microcapsule (Paşcalău *et al*, 2016), liposome (Jin *et al*, 2016), micro sphere (Deng *et al*, 2015), micro emulsion (Sobh *et al*, 2015), solid dispersion (Nguyen *et al*, 2015), tablet (Hani *et al*, 2016), inclusion compound (Du*et al*, 2020), etc. However, curcumin is insoluble in water and instable that it is meaningful to design a formulation which can improve the solubility and stability of curcumin. Thus, we prepared niosomes which could overcome the shortcoming of curcumin being insoluble in water, improve the stability of curcumin, and be targeted the liver and spleen to improve the therapeutic effect of curcumin on liver cancer and hepatitis.

In this study, curcumin niosomes with high encapsulation efficiency were prepared by film dispersion method, and the preparation of niosomes was optimized by single-factor experiments and central composite design. Moreover, *in vitro* release experiments were studied and the pharmacokinetics evaluation was executed in New Zealand rabbits.

MATERIALS AND METHODS

Materials

Span80, cholesterol, absolute ethanol were obtained from Sinopharm Chemical Reagent Co., Ltd. Curcumin was obtained from Shenzhen Heng Seng Biological Technology Co., Ltd. High performance liquid chromatography (HPLC) grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). The nitrendipine (internal standard) standards were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

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Procedures

Preparation of curcumin niosomes

The curcumin niosomes were prepared by film dispersion method (Rui Li *et al*, 2017). A certain volume of the span 80 ethanol solution, cholesterol ethanol solution and curcumin ethanol solution were put in a dry round bottom flask, rotary evaporated to form a uniform film on the bottle wall, hydrated with PBS (pH was 6.5), ultrasound 3min, and filtered by 0.45μm membrane. Then, the curcumin niosomes were prepared.

Determination of entrapment efficiency

The encapsulated drug was precipitated by centrifugation at low speed and the niosomes remained in the supernatant after centrifugation (Xiao, 2021). 5mL curcumin niosomes suspensions and 5mL ethanol were put in 10mL centrifuge tube, mixed, ultrasonic, centrifuged at 4000 rpm for 10min and the absorbance of supernatant was determined to calculate the total amount of curcumin in the niosomes suspensions (a). 5mL curcumin niosomes suspensions was centrifuged at 4000 rpm for 10min and the absorbance of supernatant was determined to calculate the amount of curcumin encapsulated in the niosomes (b). Encapsulation efficiency was calculated as the percentage of the amount of curcumin encapsulated in the niosomes (b) relative to the total amount of curcumin in the niosomes suspensions (a).

Single factor experiment

Taking encapsulation efficiency as indicator, six factors, such as the ratio of Span 80 to cholesterol, the ratio of cholesterol to curcumin, PBS volume, hydration speed, hydration time, hydration temperature were investigated.

Central composite design experiment

In the study, a 5^3 central composite face-centered design was used for optimizing of the curcumin niosomes. Three main variables, i.e., the PBS volume (X_1, mL), the hydration speed (X_2, r/min) and the ratio of Span 80 to cholesterol (X_3, g/g) were selected and studied at five different levels (-1.732, -1, 0, 1 and 1.732). With entrapment efficiency as the response, experiments were carried out according to the experimental design shown in table 1. The standard deviation (SD) was below 0.90%.

The statistical model was analyzed by Statistical 6.0 software to validate responses to variables within the design space. Finally, entrapment efficiency determination experiments under optimized conditions were conducted to compare with the predicted values.

Establishment of regression curve

Certain curcumin was put in 10mL volumetric flasks, and absolute ethanol was added to prepare 2, 3, 4, 5, 6μg/mL standard solutions. The standard curve was established and linear regression was carried out.

Size distribution and Zeta potential of curcumin niosomes

The average particle size and Zeta potential of curcumin niosomes were measured by BI 90plus laser particle size analyzer after dilution of the niosomes with distilled water at 25°C.

Electron microscopic morphology of curcumin niosomes

After shaking, the appropriate amount of the niosomes suspensions were dropped onto the sample holder with a dropper. The liquid was dried and sprayed with gold, and the morphology of the niosomes was observed under scanning electron microscope.

Stability experiment

The niosomes were sealed and kept in dark place, and the stability of niosomes at 25°C and 4°C was investigated. The encapsulation efficiency was measured at 0, 7, 15 and 30 days and the appearance of the niosomes was observed and recorded.

In vitro release

Curcumin niosomes and curcumin suspensions (after adding PBS pH6.5 containing 0.5% CMC to curcumin, vortexed for evenly blending) were respectively placed in dialysis bags. In vitro dissolution study was carried out according to the Chinese Pharmacopoeia 2015 apparatus II method (paddle method) at 75 rpm in 900mL artificial gastric juice with 0.4% (w/v) Tween-80 and artificial intestinal juice with 0.4% (w/v) Tween-80 at (37+1)°C. 6 mL sample was withdrawn and 6mL fresh medium was replenished at 1, 2, 3, 4, 5, 6, 7 and 8h. Samples were filtered with a micro porous membrane (0.45μm) and measured at 429 nm on a UV spectrophotometer. All experiments were performed in triplicate.

Ethical concerns

All the animal experiments were approved and supervised by the Medical Ethics Committee of Affiliated Hospital of Qingdao University (QYFY WZLL 27291), the house and handle of animals were in accordance with the University Unit for Laboratory Animal Medicine guidelines. All New Zealand rabbits were raised in individual cages at a temperature of (20±2)°C and a humidity of (65±5)%. (Zheng, 2022)

In vivo evaluation of the curcumin niosomes in rabbits

Six New Zealand rabbits weighing 2.8-3.2kg were selected for *in vivo* study and divided into two groups of control and test at random. The rabbits were fasted 12h with free access to water. The control group and test group was intragastric administered with curcumin niosomes and curcumin suspensions, respectively. The curcumin dose of each New Zealand rabbit in both groups was 100mg/kg. Blood samples taken from ear vein were placed in heparinized tubes and centrifuged at 4000 rpm for 10 min. Then, the plasma was separated.

200µl of nitrendipine solution (100µg/mL) was added into test tube and dried under nitrogen, then 1mL of plasma and 2mL of acetonitrile were added and vortexed for 3 min, followed by centrifugation (10,000 rpm, 10min). The supernatant was dried under nitrogen at room temperature and the residue was reconstituted in 50µl methanol and vortexed for 1 min to determine.

Chromatography conditions were as follows: the Dionex Ultimate 3000 liquid chromatography with a reversed-phase column (Hypersil ODS-2, 4.6×200 mm i.d. 5 µm); UV detection at 426 nm; the mixture of methanol- water-acetic acid (60:38:2, v/v/v) as the mobile phase; 0.6 mL/min as flow rate; 25°C as column temperature. 3p87 software was used for pharmacokinetic data analysis.

STATISTICAL ANALYSIS

The results were analyzed by ANOVA using 3p87 Statistical software (version 6.0) and the standard of significant was $p<0.05$.

RESULTS

Single factor experiment

The effect of the ratio of Span80 to cholesterol

As shown in fig. 1A, with the increase of the ratio of Span80 to cholesterol, the entrapment efficiency of niosomes increased first because the Span 80 could encapsulate more curcumin. However, the entrapment efficiency of curcumin niosomes prepared with larger ratio of Span80 to cholesterol decreased because the amount of cholesterol which could reduce the fluidity of membrane was so little that the fluidity of membrane was too strong to form stable niosomes. Thus, 3:1-5:1 was selected as the ratio of Span80 to cholesterol.

The effect of the ratio of cholesterol to curcumin

As a commonly used membrane additive for the preparation of niosomes, cholesterol could reduce the fluidity of the membrane, increase the rigidity, improve the stability and reduce the permeability. As shown in fig. 1B, with the increase of the amount of cholesterol, the entrapment efficiency of niosomes increased first because the membrane was more stable and the permeability decreased. However, when the curcumin niosomes were prepared with excessive cholesterol, the fluidity of the membrane was too low, the rigidity was too strong and it was not conducive to the formation of niosomes, causing the decrease of encapsulation efficiency. Thus, 5: 1 was selected as the ratio of cholesterol to curcumin.

The effect of the PBS volume

When the PBS volume was too small, the dispersion of niosomes was low, niosomes adhered to each other, Span80 on the membrane adhered some curcumin which was easy to separate from the membrane, causing the low encapsulation efficiency. As shown in fig. 1C, with the increase of the PBS volume, the entrapment efficiency of niosomes increased because the dispersion of niosomes was better and Span 80 could well encapsulate curcumin. However, when the curcumin niosomes were prepared with excessive PBS volume, encapsulation efficiency decreased. Thus, 10-30mL was selected as the PBS volume.

The effect of the hydration speed

When the hydration speed was too low, the film could not be evenly distributed in the PBS in the hydration process, there was still some film sheet, and some curcumin was not encapsulated in the membrane, causing the low encapsulation efficiency. As shown in fig. 1D, with the increase of the hydration speed, the film was better dispersed to form niosomes and the encapsulation efficiency was improved. However, when the hydration speed was too high, the niosomes were destroyed in the hydration process and the encapsulation efficiency decreased. Thus, 200-600r/min was selected as the hydration speed.

The effect of the hydration time

When the hydration time was too short, the member could not completely encapsulate curcumin that the encapsulation efficiency was low. As shown in fig. 1E, with the increase of the hydration time, the entrapment efficiency of niosomes increased first because the member could encapsulate more curcumin. However, when the hydration time was too long, the niosomes were destroyed in the hydration process, causing the decrease of the encapsulation efficiency. Thus, 1.5h was selected as the hydration time.

The effect of the hydration temperature

As shown in fig. 1F, when the temperature was low, the influence of hydration temperature on entrapment efficiency was less. With the increase of the temperature, the encapsulation efficiency increased slightly. However, when the temperature was too high, the capsule material was easy to precipitate and the encapsulation efficiency was decreased. Thus, 50°C was selected as the hydration temperature.

Central composite design

The experimental results after performing 20 runs of the central composite design (CCD) were shown in table 2.

The values for the entrapment efficiency of the niosomes were analyzed by ANOVA. In the process of binomial fitting, the term of $P>0.05$ (b7, b8, b9) was abandoned and the binomial fitting significance test and variance analysis were shown in table 3. Results of linear regression and binomial fitting equations were presented in table 4. When R was closer to1.0, the regression line fitted the data better. The R values for linear regression and binomial fitting model were 0.36194012 and 0.92822976, respectively. Thus, the binomial fitting model was better.

Table 1: Coded levels and independents variables for central composition design.

Factors	Coded levels				
	-1.732	-1	0	1	1.732
X_1(mL)	10	14.2	20	25.8	30
X_2(r/min)	200	285	400	515	600
X_3(g/g)	3	3.4	4	4.6	5

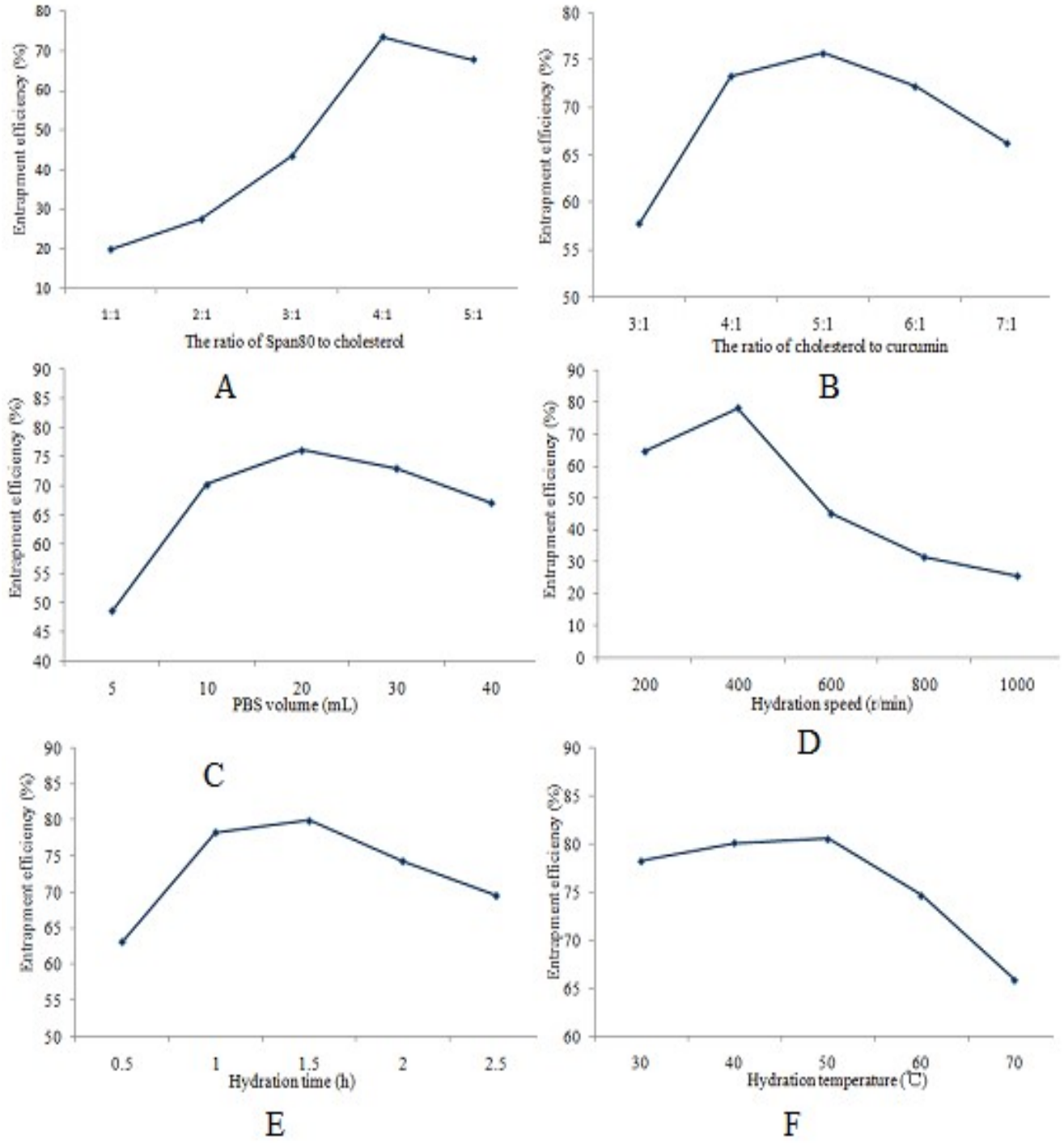

Fig. 1: Effect of the ratio of Span80 to cholesterol (A), the ratio of cholesterol to curcumin (B), PBS volume (C), hydration speed (D), hydration time (E), hydration temperature (F) on entrapment efficiency

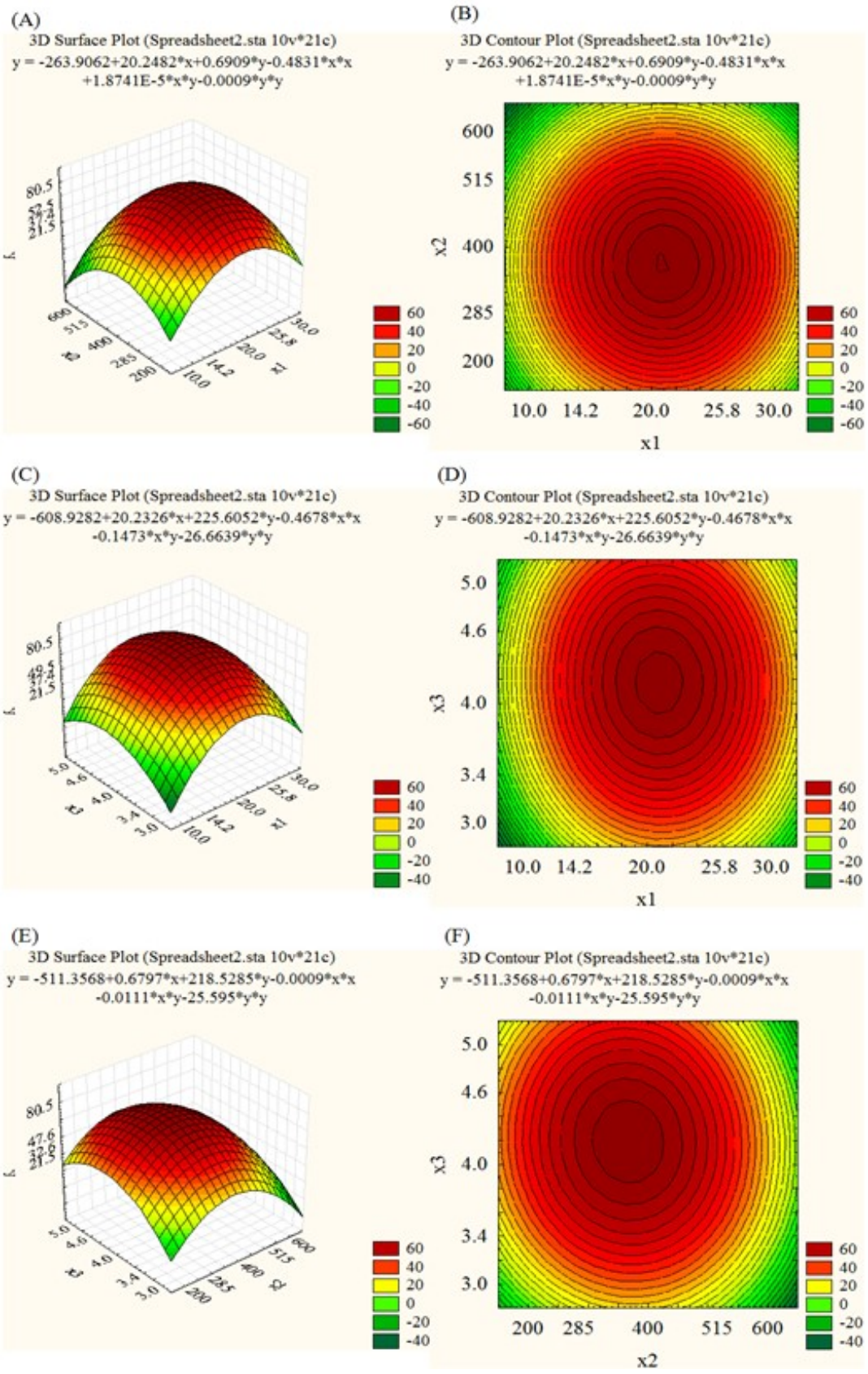

Fig. 2: Results of central composite design.

Interaction influencing determination of optimal prescription

The 3D response surface plots showed the region of maxima (red) and minima (green) for responses. From below six figs. shown in fig. 2, we concluded that the optimum range of the three factors were 20.2~21.3mL (X_1), 355~390r/min (X_2), 3.92~4.33 (X_3) respectively.

Establishment of regression curve

The regression equation of standard curve was A=0.1052c+0.0197, R^2=0.9995.

Table 2: Central composite design of the variables with activity as response.

Run	X_1	X_2	X_3	y
1	-1	-1	-1	47.6
2	1	-1	-1	56.5
3	-1	1	-1	32.6
4	1	1	-1	49.5
5	-1	-1	1	40.6
6	1	-1	1	55.4
7	-1	1	1	30.5
8	1	1	1	37.4
9	-1.732	0	0	22.5
10	1.732	0	0	38.6
11	0	-1.732	0	52.5
12	0	1.732	0	30.5
13	0	0	-1.732	21.5
14	0	0	1.732	80.5
15	0	0	0	85.1
16	0	0	0	84.8
17	0	0	0	84.2
18	0	0	0	83.9
19	0	0	0	83.7
20	0	0	0	84.7

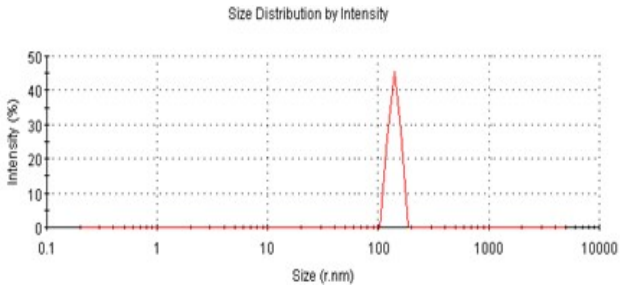

Fig. 3: Size distribution of curcumin niosomes

Particle size distribution and Zeta potential of curcumin niosomes

As shown in fig. 3, the particle size distribution of curcumin niosomes was concentrated and uniform. The average particle size was 162 nm and the polydispersity index (PDI) was 0.275.

It can be seen from fig. 4, the Zeta potential of curcumin niosomes suspensions was (-28.9+2.7) mV.

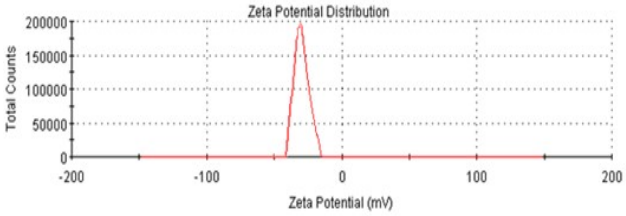

Fig. 4: Zeta potential distribution of curcumin niosomes

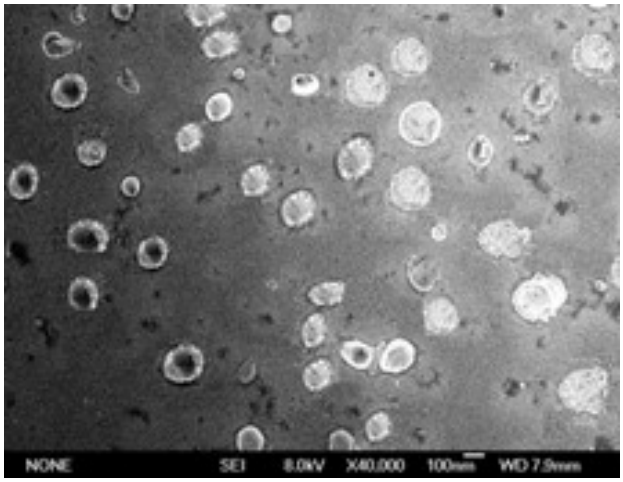

Fig. 5: Scanning Electron Microscope picture of curcumin niosomes Stability experiment

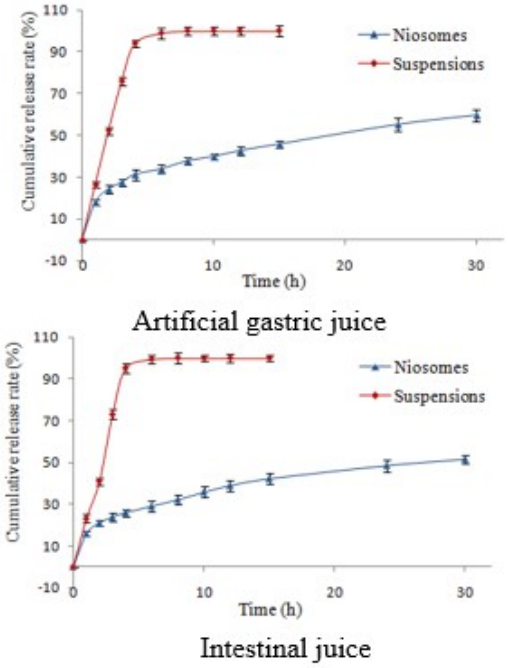

Fig. 6: *In vitro* release of curcumin from niosomes and curcumin suspensions in artificial gastric juice and intestinal juice.

Electron microscopic morphology of curcumin niosomes

The morphology under scanning electron microscope of curcuin niosomes obtained by the optimum formulation was shown in fig. 5.

Table 3: Simplified results of binomial model fitting

Parameter	Estimate	Standard	t-value	p-level	Lo. Conf	Up. Conf
b0	-818.289	140.6156	-5.81934	0.000060	-1122.07	-514.508
b1	21.625	3.2383	6.67781	0.000015	14.63	28.621
b2	0.762	0.1624	4.69077	0.000422	0.41	1.113
b3	258.485	63.4084	4.07652	0.001310	121.50	395.471
b4	-0.517	0.0800	-6.46505	0.000021	-0.69	-0.344
b5	-0.001	0.0002	-5.08645	0.000209	-0.00	-0.001
b6	-31.142	7.9032	-3.94043	0.001692	-48.22	-14.068

Table 4: Equation fitting results.

Equation type	Fitting equation	Correlation coefficient (R)
linear regression	$Y=-10.6438+0.2684X_1-0.0601X_2-0.0326X_3$	0.36194012
binomial fitting	$Y=-818.289+21.625X_1+0.762X_2+258.485X_3-0.517X_1^2-0.001X_2^2-31.142X_3^2$	0.92822976

Table 5: Stability result of curcumin niosomes (n=5)

Time (d)	4°C		25°C	
	Entrapment efficiency (%)	Appearance state	Entrapment efficiency (%)	Appearance state
0	88.5±0.23	suspensions, no stratification	88.5±0.23	suspensions, no stratification
7	87.1±0.48	suspensions, no stratification	85.6±0.33	suspensions, slight stratification
15	86.4±0.32	suspensions, slight stratification	83.8±0.85	suspensions, obvious stratification
30	84.9±0.54	suspensions, slight stratification	80.9±0.62	suspensions, obvious stratification

Table 6: Results of model fitting

Release media	Fitting method	Fitting model	Correlation coefficient (R^2)
Artificial gastric juice	Zero order release	F=0.0131t+0.2406	0.9297
	First order release	ln(1-F)=-0.0229t-0.2555	0.9739
	Higuchi	$F=0.0891t^{1/2}+0.116$	0.9925
	Hixson-Crowell	$(1-F)^{1/3}=-0.0063t+0.9158$	0.962
	Ritger-peppas	lnF=0.3389lnt-1.68	0.9953
Artificial intestinal juice	Zero order release	F=0.0117t+0.2092	0.9177
	First order release	ln(1-F)=-0.0185t-0.223	0.9551
	Higuchi	$F=0.08t^{1/2}+0.0967$	0.9906
	Hixson-Crowell	$(1-F)^{1/3}=-0.0053t+0.9269$	0.9443
	Ritger-peppas	lnF=0.3464lnt-1.8273	0.9969

Table 7: Parameters of pharmacokinetic of curcumin niosomes and curcumin suspensions.

Parameters	Unit	Niosomes	Suspensions
A	$ng\cdot mL^{-1}$	269.809±48.118	651.545±70.112**
t1/2Ka	h	0.523±0.198	0.545±0.036
t1/2Ke	h	5.162±0.886	1.436±0.091**
Tmax	h	1.868±0.378	1.226±0.012*
Cmax	$ng\cdot mL^{-1}$	183.077±2.369	221.127±5.267**
CL/f(s)	$L\cdot h^{-1}\cdot kg^{-1}$	0.057±0.004	0.121±0.005**
V/f(c)	$L\cdot kg^{-1}$	0.422±0.046	0.250±0.005**
AUC(0-∞)	$ng\cdot mL^{-1}\cdot h$	2143.830±181.845	870.234±31.590**
AUC(0-t)	$ng\cdot mL^{-1}\cdot h$	2074.989±146.690	803.475±23.335**
MRT(0-∞)	h	7.375±0.569	2.925±0.058**
MRT(0-t)	h	6.604±0.209	2.498±0.016**

*$p<0.05$, **$p<0.01$

Response optimization and validation of the model

The optimum formulation obtained was as follows: the PBS volume was 20.9mL, the hydration speed was 381r/min, the ratio of Span 80 to cholesterol was 4.2:1. The actual entrapment efficiency (88.5%) was close to the predicted value (89.3%). The obtained SD investigated for validation of experiment was 0.90%, which indicated that the formulation optimized by CCD was reliable. It implied that the method to optimize the formulation for larger entrapment efficiency was successful.

(A, B) Predicted response surface and contour plot of the cumulative effect of the PBS volume (X_1) and the hydration speed (X_2) on the entrapment efficiency (y); (C, D) Predicted response surface and contour plot of the cumulative effect of the PBS volume(X_1) and the ratio of Span 80 to cholesterol (X_3)on the entrapment efficiency (y); (E, F) Predicted response surface and contour plot of the cumulative effect of the hydration speed (X_2) and the ratio of Span 80 to cholesterol (X_3) on the entrapment efficiency (y).

It could be seen from the fig. that the curcumin niosomes were spherical, the size was similar, the distribution was uniform and there was no obvious agglomeration phenomenon of curcumin niosomes.

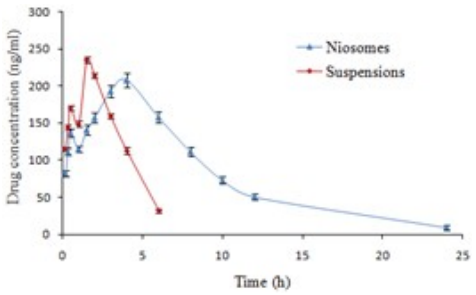

Fig. 7: Mean plasma concentration-time profiles of curcumin niosomes and curcumin suspensions (mean ±SD, n=3).

As shown in table 5, compared with the preservation at 4°C, the entrapment efficiency of curcumin nisomes preserved at 25°C exhibited a more significant decrease. When curcumin niosomes were preserved at 25°C, there was slight stratification on the seventh day and obvious stratification on the fifteenth day. However, when curcumin niosomes were preserved at 4°C, there was no obvious stratification in 30 days, indicating the stability was better. The results showed that the preservation of curcumin niosomes at 4°C was more stable than at 25°C and curcumin niosomes should be kept in cold storage (4°C).

In vitro release

The *in vitro* release of curcumin from niosomes and curcumin suspensions in artificial gastric juice and intestinal juice is shown in fig. 6.

As shown in table 6, with the correlation coefficient R^2 as index, the *in vitro* release mechanism of curcumin niosomes was in accordance with the Ritger-peppas model. In artificial gastric juice and artificial intestinal juice, the n of Ritger-peppas equation were respectively 0.3389 and 0.3464, both less than 0.45. Thus, the main release mechanism of curcumin was diffusion.

In vivo evaluation of the curcumin niosomes in rabbits

The plasma concentration-time profile and pharmacokinetic parameters of the curcumin niosomes and curcumin suspensions are respectively shown in fig. 7 and table 7. The mean residence time ($MRT_{(0-t)}$) for curcumin niosomes was 6.604±0.209h, which was significantly longer than that of curcumin suspensions 2.498±0.016 h, indicating that the retention time *in vivo* of curcumin niosomes was prolonged. The AUC_{0-t} values of the curcumin niosomes was 2074.989±146.690, which was significantly larger than that of curcumin suspensions 803.475±23.335 $ng \cdot mL^{-1} \cdot h$, the relative bioavailability of the curcumin after administration of curcumin niosomes was 258.25% compared to curcumin suspensions.

DISCUSSION

Particle size distribution was an important feature of niosomes, which directly or indirectly influenced the distribution of niosomes in vivo. When the particle size was larger than 1μm, niosomes were targeted to lung. When the particle size was 100-1000nm, niosomes were targeted to liver and spleen (Lv2015). In this study, the average particle size of curcumin niosomes was 162 nm and the polydispersity index (PDI) was 0.275. Thus, curcumin niosomes could be targeted to the liver and spleen, which indicated that curcumin could have good effect on liver cancer, hepatitis and other diseases.

An important index to evaluate the stability of niosomes was Zeta potential. When the absolute value was 5-15 mV, a certain degree of flocculation occurred. When the absolute value was approximately 30 mV, niosomes could maintain electrostatic stability and were more stable. In this paper, the Zeta potential of curcumin niosomes suspensions was (-28.9+2.7) mV, which indicated that the stability of the curcumin niosomes was better.

There was bimodal phenomenon of the plasma concentrations-time profile of curcumin niosomes and curcumin suspensions, which was consistent with the report of Chen Xi *et al* (Chen *et al*, 2012). The bimodal phenomenon was due to the forming curcumin - gluconic acid glycoside compound by the metabolism of curcumin in liver microsomes and the enhanced water solubility. With bile secretion entering intestinal tract, curcumin - gluconic acid glycoside compounds were reduced into the original drug under the action of intestinal bacterial enzyme and absorbed from the gut into the liver, resulting

in the increase of the plasma concentration and the absorption peak appeared again in the pharmacokinetic curve.

CONCLUSION

In the present study, curcumin niosomes with high entrapment efficiency were successfully formulated by film dispersion method. The niosomes could prolong the residence time of drug in body, achieve the controlled-release effect and improve the bioavailability. The curcumin niosomes are expected to become a promising drug delivery system for treating liver diseases.

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