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Preparation of curcumin ethosomes

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This study aims to prepare a novel curcumin ethosomes and investigate its properties as a drug delivery system of curcumin. A new modified method combining thin-film hydration and ultrasound was used to prepare ethosomes. Particle size was determined using Mastersizer 3000 laser diffraction analyzer. Average particle size was calculated based on the measurements of five different batches of ethosomes. Free curcumin was isolated by ultracentrifugation to determine the entrapment efficiency. High performance liquid chromatography (HPLC) was performed to determine the concentration of curcumin. The average particle size of curcumin ethosomes decreased with the increase of ethanol concentrations, whereas it increased with the increase of phospholipid concentration. The entrapment efficacy increased with the increase of ethanol and phospholipids concentration. The particle size of the curcumin ethosomes decreased with the increase of ethanol concentration, and increased with the increase of phospholipids contents. The concentration of ethanol and phospholipids showed a positive impact on entrapment efficiency. Finally, curcumin ethosomes showed high entrapment efficiency and good percutaneous permeability.

Key words: Ethosomes, curcumin, liposome, high performance liquid chromatography (HPLC).

INTRODUCTION

Curcumin (diferuloylmethane), a phenolic compound extracted from the root of *Curcuma longa*, has been widely used as a spice and coloring agent in food industry. According to our knowledge, curcumin showed anti-tumor, anti-oxidant, immunomodulatory, enhancing of apoptosis process, and antiangiogenic properties (Schaffer et al., 2011). Also, it has been reported as a mediator of chemo-resistance and radio-resistance (Bar-Seal et al., 2010). However, its limitations include easy susceptibility to oxidization and low bioavailability have prevented its further application in clinical practices.

Ethosome, a new type of liposome carrier in drug delivery system to emerge in recent years, showed characteristics of high deformability, high entrapment efficiency and percutaneous permeability through the keratoderma barrier. Compared with conventional liposome, ethosome showed more stable structure and quality which can promote the percutaneous drug absorption, increase drug storage in the skin cells and drug mobility to the cells. Additionally, it has the characteristics of prolonged action and avirulent. Therefore, it has been considered as an important drug carrier (Fang et al., 2009; de la Presa et al., 2009).

Ethosome could increase its percutaneous capability and promote the curative efficacy through gradual release of the drugs. Local administration of the drugs delivered by ethosome can avoid the first-pass effect on liver and the degradation within gastrointestinal tract, maintain the stability of the medicine in focal zone, reduce the toxicity and adverse reactions, attenuate the administration frequency, and increase the clinical efficiency and patient compliance.

In this study, ethosomes with a small particle size, even distribution and a high entrapment efficiency (EE) were

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prepared in combination of filming-rehydration and ultrasonic methods. Based on that, curcumin ethosome was produced using ethosome as a drug delivery system of curcumin.

MATERIALS AND METHODS

Preparation of ethosomes

Curcumin ethosomes was prepared by filming-rehydration and ultrasonic method (Chen Jin et al., 2010). The prepared ethosome consist of 1~3% (w/v) lecithin, 30 to 45% ethanol (v/v), curcumin (0.1%) and water. For preparation of ethosome, an amount of lecithin and that of curcumin were dissolved in a glass bottle and mixed well with a magnetic stirrer. The glass bottle was connected to an injector and sealed; thereafter ethanol was added without vaporization. The mixture was poured into a round bottom flask and a thin film was prepared using roto-evaporator. The above mentioned procedures was repeated. Double distilled water (100 ml) was added to rehydrate the film to obtain the methyl nicotinate ethosomes. Then the ethosomes were homogenized for 5 min using a sonde-type ultrasonic instrument. Subsequently, the ethosomes were filtered using a 0.22 μm disposable filter. All the procedures in this test were carried out under gaseous nitrogen at room temperature. The quality fractions of curcumin and methyl nicotinate were 0.1 and 0.2%, respectively. Curcumin was not added in the aforementioned preparation process to produce empty ethosome suspension.

Particle size of ethosomes

Particle size was measured using the Mastersizer 3000 laser diffraction particle size analyzer (Malvern, Worcestershire, UK) immediately after diluting and filtering the ethosomes with a 0.22 μm filter. The average particle size of ethosomes was calculated based on the measurements of 5 batches of ethosomes.

Entrapment efficiency of ethosomes

To obtain the curcumin ethosomes, the unentrapped part was removed using ultracentrifuge (56,000 rpm at 4℃ for 40 min) after overnight storage at 4℃. The sediments of curcumin ethosomes were kept after removal of the supernatant. High-performance liquid chromatography (HPLC) was performed to evaluate the drug absorption from the ethosomes. The entrapment efficiency (EE) was calculated according to the following formula:

$$EE = DE / (DE + DS) \times 100$$

Where DE stands for the drug content measured from the parvules; Ds stands for the drug content measured from supernatant. All the results obtained were presented as the mean±standard deviation.

High performance liquid chromatography (HPLC) analysis

HPLC analysis was performed using Dionex U3000 system. The curcumin ethosomes were separated on a μ-Bondapak C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase was 43 : 57 (v/v) methanol-water at a flow rate of 1 ml/min. The wavelength for UV detection was 219 nm. The column temperature was set at 30℃. A 20-μl sample was injected to the column. The measuring range was 0.04 AUFS. The paper speed was set at 3 mm/min. Finally, the number of theoretical plates was counted based on the peak value of curcumin.

Optimized design of ethosome prescription

The basic prescription and conditions for the preparation of curcumin ethosome were selected by single factor analysis followed by optimization by orthogonal experiment. The optimized prescription design included the concentration of phospholipid, the content of ethanol, and ultrasonic processing time, which were counted as the major investigating factors (3 levels from each factor were taken). The experiment was carried out according to the orthogonal array. The prediction schemes were presented in Tables 1 and 2.

RESULTS

Effects of ultrasonic processing time on entrapment efficiency

Table 3 summarizes the effects of ultrasonic processing time on the entrapment efficiency and particle size. The results indicated that the enhanced EE was noted with increase of the ultrasonic processing time, and reached the peak value at 8 min. No significant difference was noted in the particle size with increase of the ultrasonic processing time.

Effects of ethanol content on entrapment efficiency

Table 4 summarizes the effects of ethanol content on the entrapment efficiency, which indicated that enhanced EE was detected with the increase of ethanol content. In addition, the average particle size and the gathering probability among ethosomes decreased with increase of the ethanol content. A particle size of less than 100 nm would promote the percutaneous effect of the drug. Thus, on the premise of stable ethosome structure, ethanol content should be maximized to increase the percutaneous capability.

Optimization of preparation process of ethosomes

For the single factor analysis, phospholipids concentration (A), ethanol content (B) and ultrasonic processing time (C) was used as the major investigating factors, respectively. Three levels (A1~A3, B1~ B3, C1~C3) were set for each investigating factor. The experiment was carried out according to the orthogonal array. The entrapment efficiency (EE) obtained from various factors has been shown in Table 5. The effects of the three factors on EE ranked in an order of phospholipids concentration > ethanol content > ultrasonic time. According to the medium values, the most optimized prescription was A2B3C1, based on which the entrapment efficiency was (94.5±3.1)%.

Table 1. Optimization of curcumin ethosome preparation by orthogonal array.

Number	Phospholipids concentration (w/v, %)	Ethanol content (v/v, %)	Ultrasonic time (min)
1	1	1	1
2	1	2	2
3	1	3	3
4	2	1	2
5	2	2	3
6	2	3	1
7	3	1	3
8	3	2	1
9	3	3	2

Table 2. Optimization of curcumin ethosome preparation by orthogonal array factor-level.

Level	Phospholipids concentration (w/v, %)	Ethanol content (v/v, %)	Ultrasonic time (min)
1	1.5	35	5
2	2.0	40	8
3	2.5	45	11

Table 3. Effects of ultrasonic processing time on entrapment efficiency.

Ultrasonic processing time (min)	EE (%)	Particle size (nm)
2	52.41±2.80	77.41±7.33
5	62.55±2.63	74.32±7.02
8	66.11±1.19	80.53±8.01
11	60.10±3.61	81.87±8.13
15	56.40±1.41	70.02±5.62

Table 4. Effects of ethanol content on entrapment efficiency (n=5, mean ± sd).

Ethanol content (v/v, %)	EE (%)	Particle size (nm)
30	37.26±1.21	84.41±7.83
35	44.32±1.25	74.32±7.02
40	57.72±2.32	69.53±5.21
45	70.05±3.17	50..32±5.13
50	75.41±2.35	46.02±4.22

Evaluation of the quality of curcumin ethosomes

Ten grams of curcumin ethosomes was centrifuged at 3000 r/min for 30 min, and then incubated at 60℃ or -10℃ for 24 h. After that, the curcumin ethosomes were brought to room temperature. The ethosomes were comparatively stable and not easily delaminated or precipitated at normal temperatures as no delamination was observed. For the physical properties, the ethosomes was ivory white suspension with proper viscosity and good ductility. In addition, the colloid was smooth and evenly-distributed. After probe-type ultrasound, it turned into transparent colloid solution.

Percutaneous capability through microporous membrane (orifice diameter of 0.15 μm) after deformation under external pressure (0.1 ~ 0.3 MPa) was analyzed. The passing rate (P) was calculated according to the following formula:

$$P = V_{ethosomes} / V_{water} \times 100\%$$

where $V_{ethosomes}$ stands for the time for flexible liposome colloid solution to penetrate the filter membrane; V_{water} stands for the time for water to penetrate the filter mem-

Table 5. Optimization of curcumin ethosome preparation process.

Number	Phospholipids concentration (w/v, %)	Ethanol content (v/v, %)	Ultrasonic time(min)	EE(%)
1	1	1	1	86.22
2	1	2	2	89.53
3	1	3	3	90.71
4	2	1	2	90.91
5	2	2	3	92.02
6	2	3	1	94.15
7	3	1	3	91.64
8	3	2	1	89.79
9	3	3	2	87.01
Ij	272.96	277.95	282.64	-
IIj	289.35	283.83	280.57	-
IIIj	283.44	283.97	282.54	-
Average Ij	90.99	92.65	94.21	-
Average IIj	96.45	94.61	93.52	-
Average IIIj	94.48	94.66	94.18	-
Rj	5.46	2.01	0.69	-

brane. The passing rate increased with the increase of external pressure. The peak value of passing rate reached 91% under an external pressure of 0.3 MPa, which indicated that curcumin ethosomes showed satisfactory deformability.

Average particle size played a pivotal role in the curative effect of the medicine. In our study, five batches of curcumin ethosome were separately prepared according to the optimized prescription. The average particle size of curcumin ethosomes after dilution was 54.3±5.2 nm using laser particle analyzer (Malvern Instruments Corporate, Worcestershire, UK). Additionally, the distribution of ethosomes was found to be tight with an averaged particle size of 51.8±4.7 nm.

Measurement of curcumin content

To prepare the curcumin stock, 4 mg of standard curcumin was dissolved in methanol in a 100-ml volumetric flask. To dilute the curcumin stock, different volumes of stock were added in 10-ml volumetric flasks, and diluted with methanol. After filtering with Millipore, 10 µl from each dilution was separately injected into liquid chromatography. The peak areas were recorded respectively.

Standard curves were drawn with sample weight (µg) as X-axis and average value peak area as Y-axis. The equation of linear regression was:

$Y = 75.3261 + 211.8860X$; $r = 0.9999$.

Curcumin ethosome sample (1 ml) from 3 batches prepared according to the optimized prescription was separately added into a 100-ml measuring flask, diluted with methanol and mixed well. 20 µl of sample was injected into high-performance liquid chromatography (HPLC) column. Then the peak area was recorded and subjected to regression analysis. The contents of the 3 batches of samples were calculated as 99.01, 96.90 and 97.91%, respectively.

Measurement of entrapment efficiency

According to the optimized prescription, three batches of samples of curcumin ethosomes were prepared. The unentrapped part was removed using ultracentrifuge at 6000 r/min for 45 min after storing overnight at 4°C. The parvules of curcumin ethosome were kept after removal of the supernatant. The drug content was determined using HPLC. The entrapment efficiency was calculated with the following formula:

$EE=DE / (DE + DS) \times 100$

where DE stands for the drug content measured from the parvules; Ds stands for the drug content measured from supernatant. The average entrapment efficiency in this study was 94±3% (n = 3).

DISCUSSION

As a new type of vesicle-structured drug carrier, ethosome showed the characteristics of good deformability, high entrapment efficiency, satisfactory permeability and reliable stability, which enabled the delivery of drugs into deeper skin layers and/or the stystemic circulation. Meanwhile, it could promote the

internal transmission of lipophilic drugs *in vivo*. Furthermore, the ethosomes could deliver the drugs through the eyelets which was 1/10 to 1/5 less than its size without changing its shape under the hydration pressure (Godin and Touitou, 2003). The high concentration of ethanol contained in the ethosomes could increase the flexibility and fluidity of lipid bilayer, based on which the liposomes with ethanol could penetrate the cuticle. In addition, the interaction between ethanol and cuticle could increase the solubility of the drug, decrease the phase-transmission temperature, alternate the arrangement of lipid molecule, and increase the fluidity and flexibility of ethosome membrane. As phospholipid was apt to be dissolved by ethanol, the concentration of ethanol in ethosome should be less than 45% (Touitou et al., 2000; Barry, 2001).

The study presented the preparation of curcumin ethosomes based on optimal prescription. Single factor method was used to select the basic prescription and conditions for the preparation of curcumin ethosome. The phospholipid concentration, ethanol content and ultrasonic processing time were considered as the major investigating factors. Orthogonal experiment was carried out to select the optimized condition for the preparation of ethosomes with entrapment efficiency. After that the quality of curcumin ethosomes was analyzed. The results showed that the effects of three factors on the entrapment efficiency ranked in an order of concentration > ethanol content > ultrasonic processing time. The analysis of the average particle size using laser dynamic scattering equipment indicated that the particle size of ethosome decreased with the increase of ethanol concentration. However, its size increased with the increase of phospholipids concentration. No restrict correlation was identified between the ultrasonic processing time and the average particle size. Previous study indicated that entrapment efficiency and stability were correlated with particle size and distribution, and they could directly influence the activities of ethosomes in the organism (Esposito et al., 2004). Our study indicated that the entrapment efficiency of the drug increased with the increase of phospholipid and ethanol concentration as well as the ultrasonic processing time. In addition, the aggregation probability among ethosomes increased with increase of phospholipid concentration and decrease of ethanol concentration. As the particle size of ~100 nm showed negative effects on the percutaneous capability of the drug, phospholipid concentration must be controlled to modulate the percutaneous capability of the ethosomes. Additionally, the ethanol content should be increased as high as possible on the premise of the stability of ethosomes, in order to increase the percutaneous capability of the agent (Esposito et al., 2004).

In our study, significant decrease was noted in the particle sizes of ethosome compared with those of general liposomes. Presumably, the addition of ethanol in the prescription may give rise to a change in the nature of electrical charge, which may strengthen the space stability of vesicle and shrink its size. Curcumin is a fat soluble drug with relatively high entrapment efficiency. According to our knowledge, the entrapment efficiency is usually measured with dialysis or ultracentrifugation. Once the entrapment efficiency measured by ultracentrifugation is lower than that obtained from dialysis, it indicated that the drug had been partially lost due to the deformation and disruption of the lipid layers in the process of ultracentrifugation (López-Pinto et al., 2005). In the present study, the entrapment efficiency of curcumin ethosomes prepared with the mixture ratio of 45% (v/v) of ethanol to 2% (w/v) of phospholipids was over 85%. The ethosomes were small in size with good distribution and a high entrapment efficiency of ~95%. Results obtained from the minimal inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and antibiosis-seasoning experiments proved that the speed rate and the percutaneous capability of ethosomes was higher and the time lag was shorter, compared with those of liposomes, demonstrating the drug could exert its effects effectively in antibiosis and the treatment of deep mycotic infections (Godin and Touitou, 2005).

Conclusion

In this study, a novel ethosome with lecithin, ethanol and water was produced, which showed characteristics of well distribution, small particle size and high entrapment efficiency. The combination of the thin-film hydration and ultrasound technology made these ethosomes suitable to serve as liposome drug carriers. This study may provide a new orientation for the research and development of external curcumin agents.

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