Optimization and Characterization of Curcumin-Loaded Liposomes: Enhancing Bioavailability through Ethanol Injection Method

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Abstract: Curcumin, extracted from ginger, is renowned for its antioxidant, anti-inflammatory, anti-cancer. However, its instability and susceptibility to environmental factors lead to low bioavailability, hindering its use as an ideal oral preparation. To overcome this, this study explores the encapsulation of curcumin in liposomes to enhance its bioavailability, providing a viable drug carrier for clinical applications. Liposomes, due to their distinctive structure-activity relationship, facilitate increase their absorption and utilization in the body. This study utilized the ethanol injection method to prepare curcumin, with a focus on optimizing encapsulation efficiency, particle size, and polydispersion index intending to ascertain the most favorable preparation conditions. Extensive testing determined the optimal component content in curcumin liposomes and control tests were conducted in diverse environments to refine the data on curcumin. The optimal preparation process identified involves a cholesterol to lecithin ratio of 1:3, a curcumin to lecithin ratio of 1:12, a phosphate-buffered paline concentration of 0.2mol/L, a pH of 6.5, an addition amount of 30ml, and a water bath temperature of 45 ℃. Under these conditions, we achieved an optimal encapsulation efficiency of 77.58%, a particle size of 153.9nm, a polydispersion index of 0.180, and a Zeta potential of -11.3mV.

1. Introduction

Curcumin, a diketone compound is a naturally active constituent extracted predominantly from the rhizomes of turmeric, contributing to 3-6% of turmeric's total mass. It is renowned for its multifarious pharmacological properties including anti-inflammatory, antioxidant, and anti-cancer activities, substantiating its extensive application in the pharmaceutical sector[1]. However, the practical application of curcumin is still more problematic. Firstly, curcumin is very unstable. It is sensitive to light, with inactivation rates as high as 68.9% after 5 d of light exposure[2]. It is also thermally unstable, and the curcumin structure is destroyed when the temperature reaches above 70°C [3]. Under strong acid and alkali conditions, curcumin is easily decomposed to produce new substances[4]. It also undergoes chelation reactions with metal-ions[5]. Secondly, researchers have shown through clinical trials that the bioavailability of curcumin administered orally is very low. Micromolar curcumin can only be detected in the gut when higher doses (over 3.6g) are taken orally. Moreover, scientists detected metabolites of curcumin in peripheral blood and liver tissue, suggesting a primacy effect of curcumin[6]. Numerous strategies have been deployed by scientists to ameliorate its bioavailability, one such being the utilization of liposomes. A liposome is an intricate, ultrafine delivery mechanism primarily composed of amphoteric molecules such as phospholipids. When dispersed in water, these molecules, owing to the propensity of their hydrophobic ends to polymerize and hydrophilic ends to interact with water, form bilayered encapsulated structures, capable of interfacing with both aqueous phases and bimolecular membranes[7]. Thus, liposomes can encapsulate curcumin and protect it from degradation in vivo, thus improving its stability. Secondly, liposomes can enhance the solubility of curcumin and improve its bioavailability. Finally, liposomes can target specific tissues and cells to improve curcumin specificity. Thus, curcumin liposomes could have applications in cancer therapy, anti-inflammatory therapy, and neuroprotection. Studies have shown that curcumin-containing liposomes can enhance the anticancer effects of curcumin by improving cellular uptake of curcumin and specifically targeting cancer cells. Thus, the development of curcumin liposomes as a targeted drug delivery system will change the way traditional diseases are treated. While improving the bioavailability and therapeutic capacity of curcumin, the potential drug toxicity of curcumin will be reduced by specifically targeting patient cells. Ethanol injection is one of the methods for the preparation of liposomes, boasting advantages like simplistic procedure, manageable particle size, absence of toxic solvents, and ease of mastery over other methods such as thin film dispersion and reverse phase evaporation[8,9]. Ethanol injection is one of the

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methods for the preparation of liposomes, boasting advantages like simplistic procedure, man-ageable particle size, absence of toxic solvents, and ease of mastery over other methods such as thin film dispersion and reverse phase evaporation [10,11,12].

Acknowledging curcumin's significant potential, this research delves into its multifaceted pharmacological attributes and addresses its bioavailability issue to enhance its medical efficacy and applicability. Through advanced ethanol injection methods for preparing liposomes, we aim to optimize curcumin's encapsulation efficiency and negate the need for toxic solvents, in contrast to traditional methods like thin film dispersion. This technique not only emphasizes our dedication to improving curcumin's bioavailability but also offers a robust and efficient delivery mechanism. By thoroughly analyzing curcumin-liposome interactions and optimizing preparation conditions, we aim to clarify curcumin encapsulation intricacies and spur advancements in related pharmaceutical research. Our methodological and innovative contributions aim to significantly advance knowledge and applications of curcumin in pharmaceutical fields.

2. Materials and Methods

2.1. Synthesis of curcumin liposome

(1) Prior to the synthesis: Thoroughly cleanse and dry two 50ml beakers and place magnetic stir bars in one of them; set the water bath to preheat to 45 °C. (2) Aqueous phase: Into one beaker, incorporate 30ml of phosphate-buffered saline (PBS) solution and a trace amount of Tween-80 to facilitate solubilization, then heat to 45°C in a water bath and reserve. (3) Ethanol phase: Accurately weigh requisite amounts of lecithin, cholesterol and curcumin at the same time, document the values, and transfer them to the second beaker, followed by the addition of anhydrous ethanol solution, and subsequent sonication in a water bath until dissolution is achieved, yielding an orange transparent solution. (4) Synthesis: Equip a No. 5 syringe with the ethanol phase and gradually introduce it into the aqueous phase, maintaining con-trolled stirring of the aqueous phase simultaneously. Upon complete integration of the organic phase into the aqueous phase, undergo a hydration period of 60 minutes and allow it to cool to ambient temperature to achieve a homogeneous liposome solution.

2.2. Single factor investigation

In the synthesis of curcumin liposomes, the impact of the fabrication procedure on the encapsulation efficiency of curcumin was investigated through the modulation of individual variables. Specifically, (1) the PBS was adjusted to pH levels of 5.5, 6.5, and 7.5, (2) the mass ratios of curcumin to lecithin were designated at 1:8, 1:12, and 1:15, (3) the concentrations of Tween-80 was 30, 100, 200, and 1200 mg, and (4) the ethanol content was manipulated to constitute 3%, 15% and 20% of PBS volume[10].

2.3. Assessment of entrapment efficiency in curcumin liposomes

The formulation of the curcumin standard curve was guided by meticulous analysis of the experimental outcomes and is substantiated by extensive literature. Under the specified experimental conditions, the maximum absorption wavelength of curcumin is 425nm. Therefore, the curcumin solution prepared by curcumin standard was measured in 0.1,0.2,0.3,0.4 and 0.5mL respectively, put into 10mL brown volumetric flask and fixed volume with anhydrous ethanol. The absorbance of the sample was quantified in 425nm band by UV-Vis spectrophotometer. Finally, a standard curve equation was derived by plotting the concentration on the abscissa and the absorbance on the ordinate.

To assess the entrapment efficiency, 350 μL of the curcumin liposome suspension was accurately pipetted and transferred to a 5 mL centrifuge tube using a precision liquid transfer instrument. The tube was then filled to the 3.5 mL mark with anhydrous ethanol, followed by thorough vortexing. The total absorbance, A_{total} ,of curcumin was then measured at 425nm using UV-spectrophotometer. In parallel, another 1 mL curcumin liposome suspension emulsion was put into 1.5mL centrifuge tube and centrifuged at 10000 rpm centrifugation 30min. Subsequently, 350 μL of supernatant was taken into 5ml centrifuge tube, anhydrous ethyl alcohol was added to 3.5ml, and shaken well. The absorbance, A unencapsulated , at the maximum wavelength by UV-spectrophotometer. According to the standard curve, A_{total} and $A_{unencapsulated}$ and the corresponding actual concentration C_{total} and $C_{unencapsulated}$. If the absorbance is linear with the concentration, the value of entrapment efficiency can be directly obtained by the ratio of absorbance. The general formula of entrapment efficiency is as follows:

$$\text{Entrapment efficiency (EE) (\%)} = (M_{loading} / M_{dosage}) \times 100\% \quad (1)$$

2.4. Analysis of particle size of curcumin liposomes

Curcumin liposome suspensions with different ratios were systematically prepared, with 1 mL of each subsequently transferred to a 10 ml centrifuge tube. Curcumin liposome suspension was further diluted tenfold using PBS buffer to ensure accuracy in subsequent analyses. Under the condition of 25 ℃ and scattering angle 90°, the particle size, polydispersion index (PDI) and Zeta potential of curcumin liposome suspension were measured by Malvern nano-size Zeta potential analyzer.

2.5. Stability analysis of curcumin liposomes

Curcumin liposome suspensions were synthesized according to the optimum process, which was packed in a centrifuge tube and stored at ambient conditions for 9 days

and observed every 3 days. Its stability was evaluated according to the changes of average particle size and PDI.

3. Results and discussion

3.1. Standard curve of curcumin

The standard curve illustrated in Figure. 1 reveals the mathematical relationship for curcumin as Y=0.1541x+0.1223, and the linear regression coefficient, R^2, approximating 0.999. The standard curve has a good linear correlation, which shows that there is a good linear relationship between the concentration of curcumin standard solution and its absorbance, affirming the reliability of the established calibration curve for subsequent quantitative analyses.

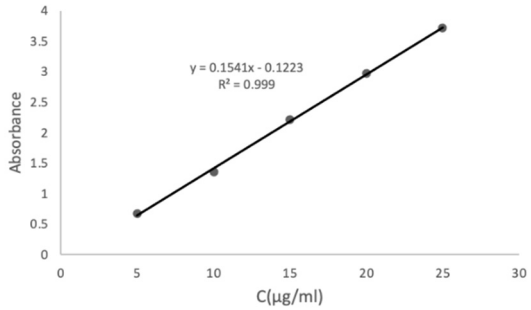

Figure 1. Standard curve of curcumin

Therefore, we can calculate the entrapment efficiency according to the absorbance:

$$\text{Encapsulation efficiency (EE) (\%)} = (A_{total} - A_{unencapsulated}) / A_{total} \times 100\% \quad (2)$$

3.2. Analysis of the results of single factor experiment

The effect of different PBS buffers on the particle size of curcumin is shown in Table 1. The results showed that changing the pH value of PBS buffer had little effect on the particle size of curcumin liposomes. However, as shown in figure 2, the entrapment efficiency of curcumin liposomes increased and then decreased with the increase of pH in PBS buffer. when pH was 6.5, the entrapment efficiency of curcumin liposomes was 77.58%. Under other pH conditions, the entrapment efficiency is: pH is equal to 5.5, the entrapment efficiency is 47%, the pH is equal to 7.5, and the entrapment efficiency is 69.1%.This is because the pH value of the aqueous solution is related to the stability of the liposome. When the solution tends to be acidic or alkaline, the water will contain more H+ or OH-. These H+ or OH- ions will hydrolyze with phospholipids. In addition, the pH value can also cause the change of the charge on the phospholipid membrane, which in turn affects the permeability of the lipid bilayer and leads to the decrease of the coated drugs. Therefore, the pH value of PBS buffer should be 6.5.

Table 1. Effect of PBS buffer on the particle size of curcumin liposomes

pH value	Appearance	Particle size (nm)	Stability (days)
5.5	yellowish emulsion	146.4	Stable within 3 days
6.5	yellowish emulsion	150.2	Stable within 3 days
7.5	yellowish emulsion	148.3	Stable within 3 days

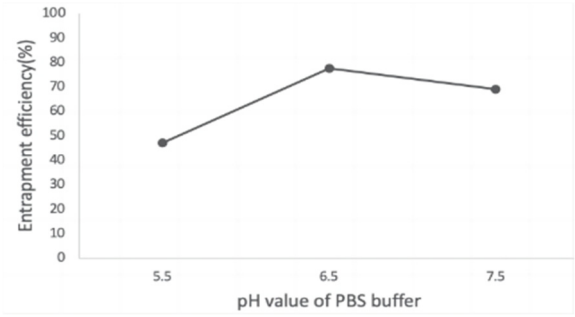

Figure 2. Effect of PBS buffer on the encapsulation rate of curcumin liposomes

The mass ratio of curcumin to lecithin was 1:8, 1:12 and 1:15 (i.e., the mass of curcumin was about 6mg, 8mg and 12mg), and different ratios of curcumin-liposome suspension emulsions were prepared to investigate the effect of the mass ratio of curcumin to lecithin on the particle size and encapsulation rate of curcumin-liposomes. The particle size results from Table 2 shows that the curcumin liposomes prepared are more stable

when the ratio of drug and lecithin is low and the particle size is large. However, as the mass of curcumin decreases, the particle size of curcumin liposomes decreases, but the stability of curcumin liposomes also decreases, resulting in flocculation. The results of the encapsulation rate are shown in Figure.3: the encapsulation rate of curcumin liposome shows an increasing and then decreasing trend with the increase of the mass of curcuma longa, and when the mass of curcumin is 8mg, the encapsulation rate is 67.2%. The other encapsulation rates were 46.4% for 6mg of turmeric quality and 23.7% for 12mg of turmeric quality. The trend in encapsulation rate may be since the size of the vesicles formed by lecithin is related to the lecithin content, and if the quality of lecithin is low, then the vesicles will become smaller and will not be able to completely encapsulate all the curcumin, resulting in a portion of the curcumin not forming into liposomes. Therefore, the mass ratio of curcumin to lecithin was selected as 1:12 by considering both particle size and encapsulation rate.

Table 2. effect of the ratio of curcumin to phospholipids on the particle size of curcumin liposomes

Ratio of curcumin to phospholipids	Appearance	Particle size (nm)	Stability (days)
1:8	yellowish emulsion	194	Stable within 3 days
1:12	yellowish emulsion	159.3	Stable within 3 days
1:15	yellow gel, partially precipitated	100.7	Significant delamination after 24h

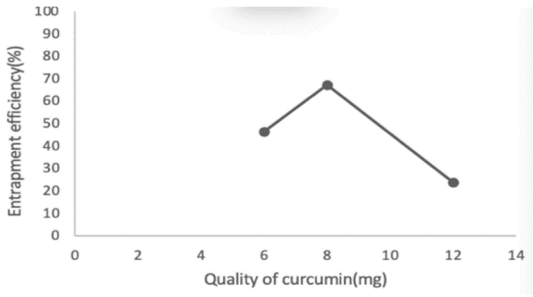

Figure 3. Effect of curcumin to phospholipid ratio on the encapsulation rate of curcumin liposomes

Curcumin liposomes were prepared by varying the dosage of Tween-80 (30mg, 100mg, 200mg, 1200mg) under the same conditions (100mg of lecithin) and the effect of Tween-80 on the particle size and encapsulation rate of curcumin liposomes was studied. The results of particle size are shown in Table 3, which indicates that the amount of Tween-80 influences the particle size and stability of liposomes. When the ratio of Tween-80 to phospholipids was greater than 2:1, the liposomes were unstable, and when their ratio was less than 2:1, the liposome particle size tended to decrease with the increase of Tween-80 dosage. The results of the encapsulation rate are shown in Figure 4, which shows that the encapsulation rate of curcumin liposomes increased significantly with the increase of the amount of Tween-80. This is since Tween-80 is a hydrophilic surfactant, which can physically adsorb onto the surface of the lipid bilayer, thereby increasing the thickness of the phospholipid membrane and increasing the space for curcumin. However, when the amount of Tween-80 was increased, the integrity of the bilayer of the lipid membrane would be damaged, resulting in the leakage of curcumin, and therefore, the encapsulation rate of the liposomes would decrease. Although the particle size of curcumin liposomes was smaller when the amount of Tween-80 was 200 mg, the encapsulation rate of curcumin liposomes was only 32.4%. Therefore, when 100mg of Tween-80 was added, the encapsulation rate was 66.4% when compared with 200mg of Tween-80, as there was no significant increase or decrease in the particle size of curcumin liposomes.

Table 3. Effect of Tween-80 addition on the particle size of curcumin liposomes

Tween-80addition(mg)	Appearance	Particle size (nm)	Stability(days)
30	yellowish emulsion	182.9	Stable within 3 days
100	yellowish emulsion	156.3	Stable within 3 days
200	yellowish emulsion	146.4	Stable within 3 days
1200	turbid yellow	499.8	Flocculation at 12 hours

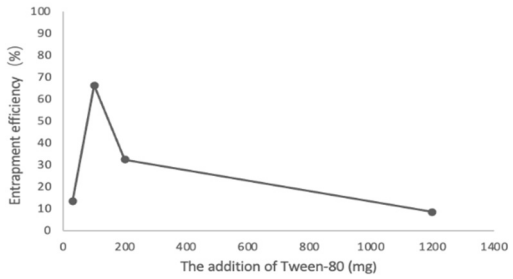

Figure 4. Effect of Tween-80 addition on the encapsulation rate of curcumin liposome

Under the same conditions, curcumin liposomes were prepared by adding 30 ml of PBS buffer at pH 6.5 with ethanol content of 3%, 15% and 20% by volume of PBS, and the effect of ethanol content on the particle size and encapsulation rate of curcumin liposomes was investigated. The particle size results are shown in Table 4, where the particle size of curcumin liposomes decreases with increasing ethanol content. This is due to the increase in the volume of ethanol, which leads to a decrease in the concentration of curcumin in the organic phase, which theoretically reduces the particle size of curcumin liposomes upon injection. However, if the volume of ethanol is too high, as shown in Figure. 5, the drug will dissolve in the ethanol, thus reducing its encapsulation rate. In addition, the more ethanol is used, it will be difficult to remove, so ethanol is 15% of the volume of PBS buffer, and the entrapment efficiency is 70.6%.

Table 4. Effect of volume ratio of ethanol to aqueous phase on the particle size of curcumin liposomes

The ratio of ethanol to water phase	Appearance	Particle size (nm)	Stability(days)
3%	yellowish emulsion	194	Stable within 3 days
15%	yellowish emulsion	159.3	Stable within 3 days
20%	yellowish emulsion	100.7	Stable within 3 days

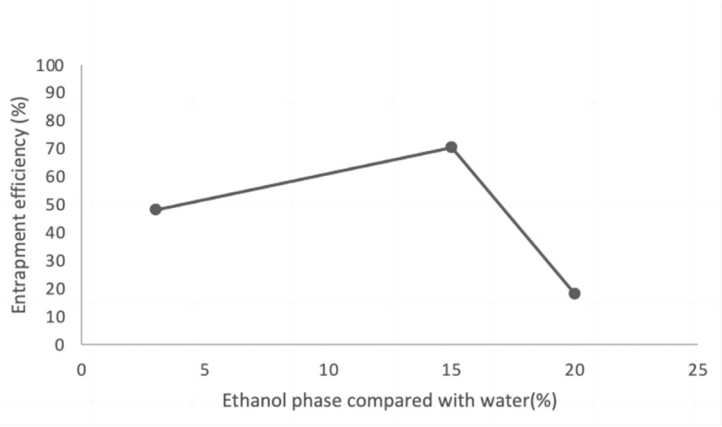

Figure. 5 effect of volume ratio of ethanol to aqueous phase on entrapment efficiency of curcumin liposomes

3.3. Curcumin particle size distribution and Zeta potential

The overall particle size distribution of curcumin liposomes ranged from 140 to 150 nm, with a PDI of 0.18 to 0.2, indicating a uniform distribution of the liposome particle size. The zeta potential was (-11.3 ± 1.56 mV) and was normally distributed, indicating that the liposomes were negatively charged, and that the higher the potential, the higher the repulsive force between the liposomes. The higher the potential, the more repulsive the liposomes are. Therefore, a large negative Zeta potential will inhibit the aggregation of liposomes and improve their stability to a certain extent. In addition, the wall material of curcumin liposomes is lecithin, which has a negative charge, which causes static repulsion between liposomes and changes the surface charge of liposomes, so that liposomes are not easy to polymerize and have better stability[11].

3.4. Stability analysis of curcumin liposomes

The stability of curcumin liposomes prepared with 100mg of lecithin, 33mg of cholesterol, 100mg of tween, 8mg of curcumin, and anhydrous ethanol at 15% of the volume of PBS (4.5mL) was observed. The particle size of curcumin liposomes at 25°C showed a tendency to increase and then decrease with the increase of storage time, increasing from 146.4 nm to 171.2 nm on the third day, and then decreasing to 87.2 nm on the ninth day. This is because liposomes are affected by the temperature of the system during storage, and phospholipids may tend to fuse with each other and aggregate, resulting in larger particle sizes. However, as time increases, the liposome membrane may

disintegrate due to oxidation and the liposomes become fragmented, resulting in a smaller mean particle size [12].

4. Conclusions

This paper has rigorously explored the optimal scheme for formulating curcumin liposomes, revealing substantial insights while recognizing inherent limitations in the research scope. A precise standard curve was established for calculating the entrapment efficiency of curcumin liposomes, Y=0.1541x+0.1223, validated to be highly accurate. Optimizations were performed on various aspects of the formulation, pinpointing the optimal pH of PBS buffer to 6.5 and determining ideal ratios of lecithin to curcumin at 1:12, lecithin to Tween-80 with Tween-80 at 100mg, and ethanol to water phase at 15% of the water phase volume. Despite achieving stable particle size data between 140 and 150 nm, inconsistencies were observed in encapsulation rates, fluctuating between 65% and 75%. This variability was ascribed to uncontrollable factors and overlooked elements such as the velocity of ethanol phase injection, imprecise magnetic stirrer speeds, incomplete centrifugation processes, and unforeseen interactions affecting the entrapment rate. The study's narrow focus on curcumin nanoliposomes preparation and analysis without exploring potential applications in other pharmaceuticals or cosmetics is acknowledged as a limitation, highlighting a need for more exhaustive and diversified research to enrich understanding and applications in this domain.

References

1. Hewlings, S. J. and Kalman, D. S., "Curcumin: A review of its' effects on human health," Foods, 6, 92 (2017).
2. Wang, X., Chen, L., and Shi, W., "Photostability study of curcumin analogues," Journal of Anhui University (Natural Science Edition), (3) (2012).
3. Qi, L. and Wang, J., "Study on the stability of monomeric curcumin," Food Industry Science and Technology, (1) (2007).
4. Cui, J., Li, Z., and Hong, X., "Free radical bioantioxidants and diseases," Journal of Tsinghua University (Natural Science Edition), (6) (2000).
5. Li, B., Li, J., Qian, H., et al., "Progress of research on the inhibitory effect and mechanism of curcumin on metal toxicity," Health Research, (3) (2011).
6. Yu, M. R., Jiang, F. S., and Ding, C. S., "Advances in the study of curcumin," Chinese Herbal Medicine, 40(5), 828-831 (2009).
7. Lazar, A. N., Mourtas, S., Youssef, L., Parizot, C., Dauphin, A., Delatour, B., Antimisiaris, S. G., and Duyckaerts, C., "Curcumin-conjugated nanoliposomes with high affinity for Aβ deposits: possible applications to Alzheimer disease," Nanomed. Nanotechnol. Biol. Med., 9, 712-721 (2013).
8. Justo, O. R. and Moraes, Â. M., "Analysis of process parameters on the characteristics of liposomes prepared by ethanol injection with a view to process scale-up: Effect of temperature and batch volume," Chemical Engineering Research and Design, 89(6), 785-792 (2011).
9. Pando, D., Matos, M., Gutiérrez, G., and Pazos, C., "Formulation of resveratrol entrapped niosomes for topical use," Colloids and surfaces B: Biointerfaces, 128, 398-404 (2015).
10. Ou, C., Liang, Y., Shen, S., and Han, X., "Preparation of curcumin liposomes by ethanol injection," Southern Journal of Agriculture, (10), 1259-1262 (2011).
11. Zhang, G.-H., Liu, H.-Q., Ye, Q., and Cheng, X.-W., "Examination of factors affecting the particle size distribution of docetaxel lyophilised liposomes after reconstitution," Chinese Journal of Pharmacy, (12), 976-978 (2012).
12. Geng, Y., Cao, D., Nie, X., and Yang, B., "Effects of oxidation on liposome membrane properties," Food Industry Science and Technology, (07), 64-67 (2013).