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Formulation and Evaluation of Curcumin-Loaded Liposomal Nanoparticles for Enhanced Bioavailability and Targeted Anti-inflammatory Therapy

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Abstract

The objective of the present investigation was to formulate liposomes of curcumin using quality by design (QbD) approach and evaluate the formulation for various parameters. A D-optimal experiment design with two independent and two dependent variables was used to optimize the formulation with the best QTPP. The particles of the optimized liposome were found to be having an average particle size of 168.4 nm with a poly dispersity index of 0.421 and a zeta potential of -18.1 mV. The entrapment efficiency was found to be 72.22 ± 0.896 % (n=3). The *in vitro* release showed that the optimal liposomal formulation released only 72.48 ± 0.832 % curcumin after 72 h. It was found that the liposomal formulation presented DPPH radical scavenging action equivalent to pure curcumin. The inhibition of albumin denaturation of the liposomal curcumin as also equivalent to pure curcumin. The ability of the liposome to combat inflammation on oral administration was compared with that of pure curcumin to ascertain its applicability using FCA induced arthritic model in rats. It was found that the liposomal formulation was able to inhibit more than 50% inflammation on the 21st day compared to 63% by pure curcumin.

Keywords: Curcumin, liposome, anti-oxidant, anti-inflammatory, optimization

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1. Introduction

Several reports related to curcumin are available and all these reports reveal that curcumin has humongous therapeutic potential [1]. The ability to avenge cancer has been the most widely investigated potential of curcumin. It has been found to have effect on almost all the known chemotherapeutic targets of cancer [2,3]. Inspite of all the potential, curcumin could not be marketed as a drug owing to its poor systemic bioavailability as well as stability issues. Several reports have been made that describe that the formulation of nanoparticles could be a viable method to improve the bioavailability of drugs [4]. Over the last few years several nanoformulations of curcumin have been reported each claiming to improve the bioavailability of curcumin [5-8]. It has also been reported that use of polymeric nanomaterials improves the encapsulation of curcumin and eventually improve the bioavailability of the drug. These systems although suffer from several drawbacks, such as poor physical stability, drug leakage, and the potential toxicity of the excipients. Liposomal nanoparticles (LNs) have recently been under consideration for drug delivery because they offer the possibility of modulating drug release and provide both stability and compatibility while avoiding the shortcomings of liposomes, including undesired stability problems and the potential toxicity of the materials such as polymeric nanoparticles [9]. A few investigations have also been reported related to formulation of liposomes using biocompatible and biodegradable lipid substances. Apart from physicochemical features, the intestinal permeability of a drug is another crucial factor for oral bioavailability. It was there envisioned to use liposomal nanoparticles approach as the carrier to improve the permeability of curcumin in the cells and hence the oral bioavailability. The lipids used to encapsulate curcumin into LNs are likely to improve the aqueous dispersibility and stability of curcumin, prolonging its efficay and cellular uptake and enhancing its bioavailability.

2. Material and Methods

Curcumin was purchased from Oxford Fine Chemicals, Mumbai. Phosphatidylcholine was obtained from Himedia whereas stearyl alcohol was purchased from Suvidhinath Laboratories. All other solvent, reagents and chemicals were purchased from Loba, CDH and Rankem.

2.1 Preformulation Studies [10,11]

The following preformulation studies were carried out on the curcumin procured from the source. The preformulation studies were carried in order to confirm the purity and identity of the procured curcumin and also to study any possible interaction with the polymeric carrier to be used in the investigation.

2.2 Drug excipient compatibility Study

IR spectra of drug and a physical mixture of drug and lipids were obtained using FT-IR spectrophotometer. The spectra were observed for physical and chemical incompatibility amongst the drug and the lipids under study.

2.3 Calibration curve of curcumin [12]

Stock solutions of curcumin containing 100 µg/mL were prepared in methanol and its aliquots were transferred in a series of 10 mL volumetric flasks in varying fractions and their volumes were made with methanol to prepare different standard dilutions (5-25 µg/mL). The solution was scanned using UV-Visible spectrophotometer from 1100 to 200 nm and the absorption maximum (λmax) was obtained to be 421 nm. The absorption of the standard dilutions was recorded at 421 nm to construct a calibration curve of concentration against absorbance. The linearity equation ($y = mx + c$) was generated and was used to calculate the concentration of curcumin in formulations.

2.4 Design of Experiments

A D-optimal experimental design was used to optimize the identified process variables that affect the CQAs. The matrix of independent variables included the two formulation factors identified as critical for liposomal quality. These factors include phospholipid concentration (X1), phospholipid to stearyl alcohol molar ratio (X2). On the other hand, the matrix of dependent variables included EE% (%) (Y1) and liposomal size (nm) (Y2).

Table 1. Design matrix for optimization of liposome by QbD

Experiment Name	X1 (mM)	X2
CL1	70	10 to 1
CL2	10	10 to 1
CL3	40	7.5 to 1
CL4	70	10 to 1
CL5	10	5 to 1
CL6	10	5 to 1
CL7	10	7.5 to 1
CL8	70	5 to 1
CL9	40	5 to 1
CL10	40	10 to 1
CL11	70	7.5 to 1
CL12	40	7.5 to 1

2.5 Formulation of liposomes

Multilamellar vesicles (MLVs) were generated using a technique based on the established film method [12,13]. Briefly, the lipid entities (phosphatidylcholine and stearyl alcohol) were dissolved in chloroform:methanol (9:1) and the solvent evaporated on a rotary evaporator to yield a dry film as per the standard lipid film hydration method. To entrap drugs within the bilayer, the required amount of drug (1 mg) was added to the solvent mixture and subsequently hydrated as per the normal hydration method. In all cases, the film was hydrated with 2 ml double distilled water.

2.6 Entrapment Efficiency evaluation

The drug loading of liposomes was determined by measuring the non-incorporated drug present in the hydration and wash media after separation of liposomes by centrifugation (Remi) at 27200g for 30 min. All samples were diluted appropriately and the drug content of the supernatant was analysed by UV spectroscopy at 421 nm. The amount of entrapped drug was calculated by subtracting the un-entrapped drug from total amount of drug used.

2.7 Determination of vesicle size

Liposomal size was determined by dynamic light scattering method, using a Zetasizer Nano ZS analyser (Malvern Instruments Co., Malvern, UK) after the dilution of the liposomes in distilled water (1:200 v/v). All the measurements were performed in triplicate at 25 °C, with a scattering angle of 90°.

2.8 Zeta Potential

Zeta potential was measured by laser Doppler electrophoresis, using a Zetasizer Nano ZS90 analyser (Malvern Instruments Co., Malvern, UK). The measurements were performed in distilled water at 25 °C, three times for each sample.

2.9 *In Vitro* Drug Release

The release rate of drug was determined by incubating drug-loaded vesicles (after separation of non-incorporated drug) in 30 ml PBS at 37°C in a shaking (constant; 150 oscillations/min) water bath. At time intervals of 0, 2, 4, 8, 24, 48 and 72 h, the medium was centrifuged at 27200g for 30 min. The supernatant was analyzed spectrophotometrically at the 421 nm wavelength and the amount of drug released was assayed by comparison with a calibration curve for drug.

2.10 Stability studies

Liposomal size and drug retention were used as parameters to preliminarily indicate the physical stability of liposomes. The protocol was adapted from du Plessis et al [30] and Vangala et al [12]. The stability of formulations, with respect to retention of the entrapped drug and changes in the size distribution, was determined by incubating vesicles (after separation of the free drug) in 10 ml PBS at 4 and 25°C. At time intervals of 0 (immediately after preparation), 7, 14 and 28th day samples

were centrifuged to separate loaded from 'free' drug, and supernatants analyzed spectrophotometrically at 421 nm. The amount of drug released was assayed by comparison with a calibration curve for drug.

2.11 DPPH radical scavenging Assay

1mM solution of DPPH solution was prepared in ethanol. 1.5ml of the liposome was added to 1.5 ml of DPPH solution. The absorbance was measured at 517 nm against the corresponding blank solution which was prepared using 3 mL ethanol. The control sample used was 3 mL of DPPH. The assay was performed in triplicates [14].

2.12 Inhibition of Albumin denaturation

The technique of inhibition of albumin denaturation reported previously [15,16] was used with slight modifications. A solution of 1% BSA in deionized water was prepared for the test. It was prepared by dissolving 0.1g BSA in 10 mL of deionized water. The reaction vessel was filled with 200 μL of BSA, 1400 μL of PBS and 1000 μL of the test solution. Distilled water was used in the control vessels instead of test solution. The reaction mixtures were incubated at 37°C for 15 min and then heated at 70°C for 5 min. The mixtures were then allowed to cool to room temperature and the absorbance of constituent of each vessel were analyzed in UV-Visible spectrophotometer at 660 nm.

2.13 Anti-inflammatory activity

All the experiments were approved by the Institutional Animal Ethical Committee and were performed in accordance to the guidelines of the ethical committee. Healthy male albino rats (200-250 g) were used in the study. Animals were maintained on standard pellet diet with free access to water in a 12-h light and dark cycle. All the animals were fasted overnight with access to water *ad libitum* before dosing. For assessing the anti-inflammatory activity and pharmacokinetic parameters of the liposomal formulation, Freund's complete adjuvant (FCA) induced arthritis model in rats was used [17]. Animal were divided into three groups of five rats each. Group 1 served as disease control; group 2 was administered liposome per oral; group 3 was administered curcumin capsule per oral (BAPS Amrut Curcumin capsule). In order to induce experimental arthritis, 0.1ml of FCA was injected into sub-plantar region of left hind paw of each rat on day 1st. Dosing of all the animal groups was started from day 12th once in a day p.o., after induction of arthritis. The inflammation was monitored by measuring paw volume with the help of plethysmometer.

3. Results and Discussion

3.1 Preformulation Studies

The results of organoleptic characterization and melting point are presented in table 2.

Table 2. Organoleptic properties of Curcumin

Test	Specification	Observation
Color	Orange- yellow needles	Pale Yellow
Odor	Characteristic	Characteristic
Taste	Bitter	Bitter
Melting Point	183°C	181-185°C

3.2 FT-IR study

The FT-IR spectrum of curcumin (Figure 1), and a physical mixture of curcumin, stearyl alcohol and phosphatidylcholine were obtained and observed for any deletion of the peaks of the pure drug.

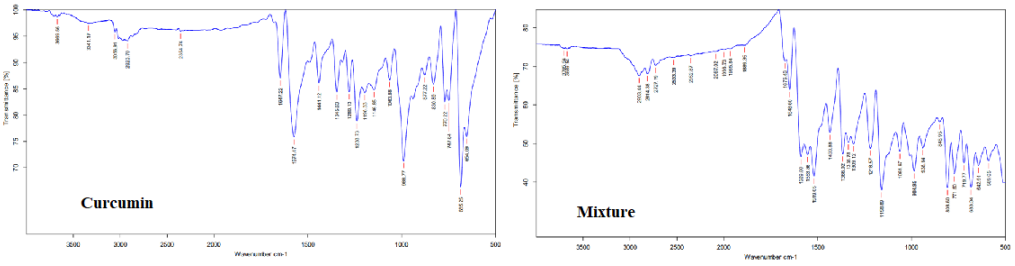

Figure 1. FTIR Spectra of curcumin and physical mixture

3.3 Calibration curve of curcumin

The calibration curve of curcumin was prepared in methanol using UV-Visible spectrophotometer at 421 nm by plotting the absorbance against concentration (Figure 2).

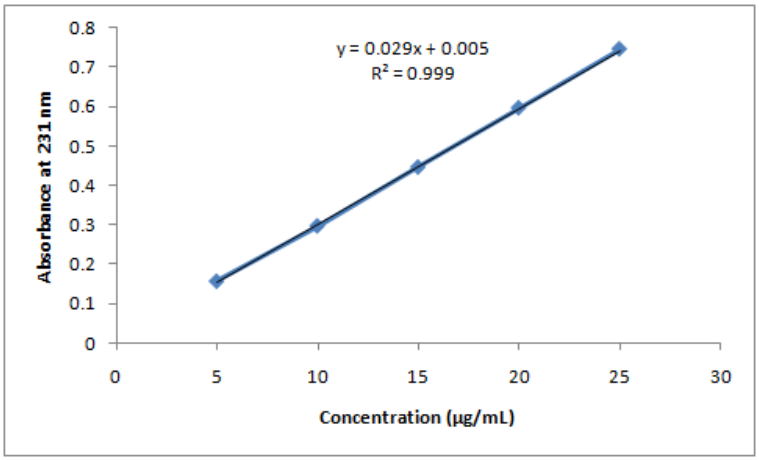

Figure 2. Calibration curve of curcumin in methanol

3.4 Optimization of formulation

The DOE was done using Design Expert 7.0.0 trial version using D-optimal design with two independent variables and two dependent variables. The result of EE% and particle size were

statistically analyzed in order to study the influence of the independent variables of them (Table 3).

Table 3. D-optimal design results

Experiment Name	PL (mM)	PL to SA ratio	EE%	Particle size
CL1	70	10:01	48.95	225
CL2	10	10:01	68.5	190
CL3	40	7.5:1	62.75	204
CL4	70	10:01	49.15	182
CL5	10	05:01	66.8	195
CL6	10	05:01	62.05	191
CL7	10	7.5:1	59.18	212
CL8	70	05:01	41.55	207
CL9	40	05:01	56.4	185
CL10	40	10:01	69.85	171
CL11	70	7.5:1	44.18	204
CL12	40	7.5:1	64.56	215

The effect of phospholipid concentration and the ratio of phospholipid to stearyl alcohol was statistically validated using ANOVA and the 2-factorial model depicted a significant F-value of 38.53 for entrapment efficiency. The model presented a regression coefficient value of 0.986 and adequate precision value of 16.724. The adequate precision measures the signal to noise ratio and a ratio greater than 4 is desirable. The optimization was done with respect obtaining with highest entrapment efficiency percent and lowest particle size of the liposomes. A total of 8 solutions were obtained of which the solution with 40mM phospholipid and 10 to 1 ratio of phospholipid to stearyl alcohol was having the highest desirability (0.521). It was selected as the optimized formulation in the design space (Figure 3).

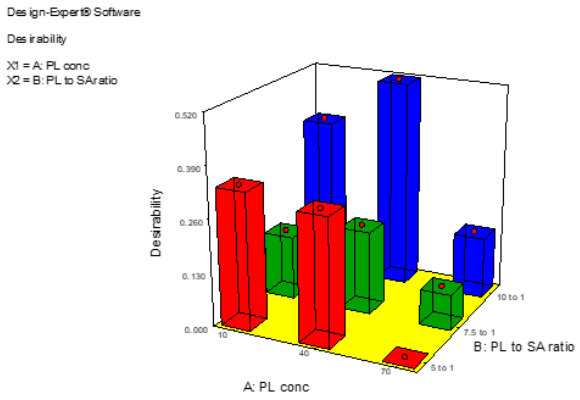

Figure 3. 3D surface plot of desirability of the soluions

3.5 Evaluation of the optimized formulation

The formulation using the optimized conditions was evaluated for entrapment efficiency, particle size, zeta potential and *in vitro* release of curcumin.

3.5.1 Particle size and zeta potential

The particle size and zeta potential were studied using Malvern zeta sizer and the particles were found to be having an average particle size of 168.4 nm with a poly dispersity index of 0.421 (Figure 4). The zeta potential of the formulation was found to be -18.1 mV (Figure 5). Though the value was not very optimum, yet values around 20 mV are considered to provide sufficient repulsion among the particles for preventing aggregation. The high poly dispersity index of the particles could be attributed to the low zeta potential of the formulation.

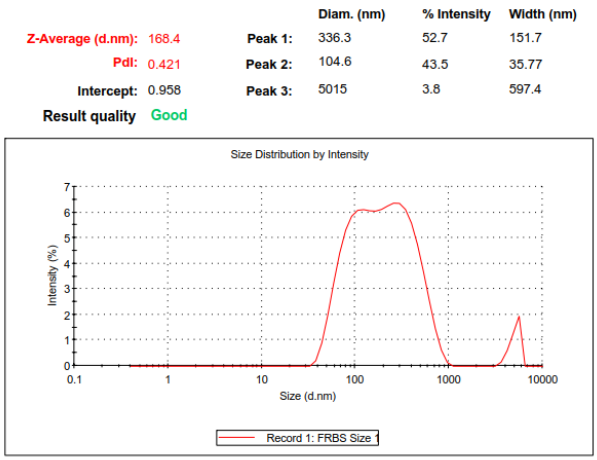

Figure 4. Particle size by intensity of optimized formulation

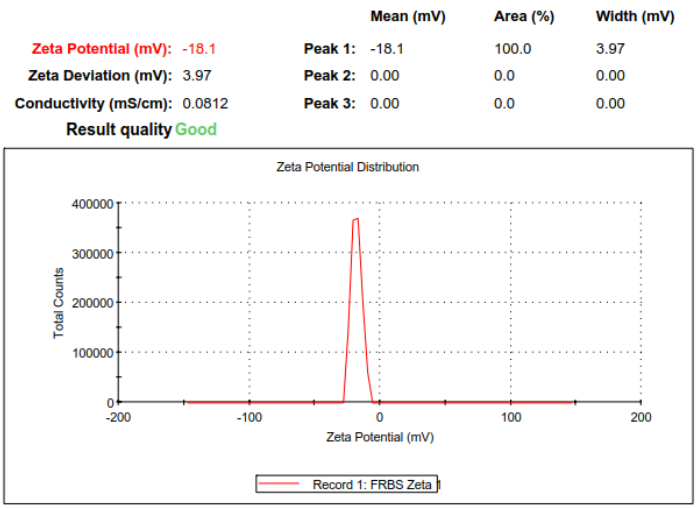

Figure 5. Zeta potential of the optimized formulation

3.5.2 Entrapment efficiency

The percentage of curcumin encapsulated or entrapped in the core of the liposomal formulation was calculated by studying the amount of non-incorporated curcumin. The entrapment efficiency was found to be 72.22 ± 0.896 % (n=3). The high incorporation of drug was beneficial as a higher EE% is associated with a reduced drug loss during the manufacturing process and with a low-cost production.

3.5.3 In vitro drug release

The release of curcumin was studied using diffusion method by calculating the amount of drug in the solution at predetermined time intervals. As the attribute required in the formulation was a prolonged duration of action of the drug, the drug release was studied up to 72 h. The cumulative amount of curcumin released from the liposome was calculated and plotted as a function of time (Figure 6).

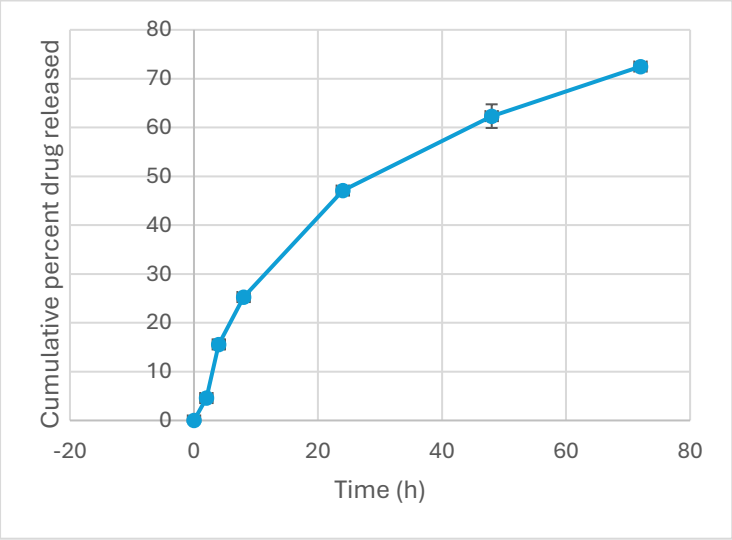

Figure 6. Plot depicting a steady release of curcumin from the liposome

The *in vitro* release showed that the optimal liposomal formulation released only 72.48 ± 0.832 % curcumin after 72 h. The amount of drug that released in the initial hours of the study increased rapidly whereas an almost plateau was attained post 48 hours of the study. The in vitro release study proved a continuous and sustained release of curcumin from the optimal formulation, for at least 72 h, fulfilling the desired attribute in the formulation as mentioned in Table 1.

3.6 DPPH radical scavenging

The absorbance of control (DPPH + methanol) as well as test solutions of liposome and curcumin was measured at 517 nm using UV-visible spectrophotometer and the % DPPH inhibition was measured (Table 4).

Table 4. % DPPH scavenging action of liposomes & curcumin

Sample	Rep 1	Rep 2	Rep 3	Mean ± SD
Liposome	45.65	44.71	45.73	45.36 ± 0.568
Curcumin	51.60	51.14	51.38	51.37 ± 0.226

The result revealed that the antioxidant action of curcumin was preserved in the liposomal formulation.

3.7 Inhibition of albumin denaturation

Protein denaturation has been significantly correlated with the occurrence of the inflammatory response and may lead to various inflammatory diseases including arthritis. It has been said that tissue injury might be due to denaturation of the protein constituents of cells or of intercellular substance. Hence, the ability of the test compounds to inhibit the denaturation of protein signifies obvious potential for anti-inflammatory activity (Table 5).

Table 5. Inhibition of albumin denaturation by liposome and curcumin

Sample	Rep 1	Rep 2	Rep 3	Mean
Liposome	61.56	63.45	64.11	63.04 ± 1.323
Curcumin	67.49	68.56	68.15	68.06 ± 0.539

***3.8 In vivo* anti-inflammatory study**

The ability of the liposome to combat inflammation on oral administration was compared with that of pure curcumin to ascertain its applicability using FCA induced arthritic model in rats. The ability to reduce inflammation is reported in table 6.

Table 6. Anti-inflammatory activity of liposome and curcumin

Group	Change in paw diameter (mm) [% inhibition of edema]			
	0th day	**7th day**	**14th day**	**21st day**
Control	0.895 ± 0.022	1.178 ± 0.035	1.802 ± 0.063	2.178 ± 0.054
Curcumin	0.894 ± 0.007	1.126 ± 0.026	1.134 ± 0.063	0.802 ± 0.065
	-	4.41%	37.06%	63.17%
Liposome	0.891 ± 0.004	1.168 ± 0.030	1.31 ± 0.067	1.074 ± 0.039
	-	0.85%	27.30%	50.68%

It was found that the liposomal formulation was able to inhibit more than 50% inflammation on the 21st day compared to 63% by pure curcumin.

4. Conclusion

The QbD approach proved to be a key element in curcumin liposome development, by providing information regarding the impact of the formulation factors and process parameters on the CQAs of the liposomes. The developed liposomal formulation presented a release of curcumin for at least 72 hours, suggesting an improved half-life, and bioavailability of the drug. The diffusion method used for assessing the drug release simulates drug release by oral administration and hence the liposome could be believed to be administered orally and fulfil all the QTPPs. The study also establishes the use of stearyl alcohol as a prominent replacement of traditionally used cholesterol in formulation of liposomes.

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