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Original Article

Development, Characterization and Evaluation of Cubosomes Loaded Smart Gel for the Treatment of Osteomyelitis using 3^2 Factorial Design

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ABSTRACT

Aim: The main aim of present study is to formulate cubosomes containing curcumin. Curcumin is a BCS class IV drug which has clinically proven good anti-bacterial property. **Materials and Methods:** Cubic nanoparticles were prepared by hydrotrope dilution method. Cubosomes were formulated with the glyceryl monooleate and pluronic F-127 as a lipid and surfactant respectively, the obtained formulations were characterized for particle size, entrapment efficiency and zeta potential. Design of Experiment (DoE) technique was used for deriving number of experiments and obtain an optimised formulation, which is further incorporated into smart gel and the smart gels were evaluated for gelation time, gelation temperature, viscosity and *in vitro* drug release. **Results:** The particle size of the optimized formulation of cubosomes was 186.27nm, zeta potential -17.5 mV and greater entrapment efficiency (71.24%). *In vitro* studies were carried out for smart gels loaded with pure drug and optimised formulation (F7) showed good sustained release activity. **Conclusion:** Hence the optimised formulation (F7) of smart gel shows good potential for treatment of osteomyelitis.

Keywords: Osteomyelitis, Cubosomes, Smart gels, Curcumin, Design of Experiment.

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INTRODUCTION

Osteomyelitis is defined as an inflammation of bone, which is typically infectious in etiology. Discussion of human osteomyelitis dates to the time of Hippocrates (460–370 BC). The coining of the term "osteomyelitis" is attributed to Nelaton in 1844. Primarily bone is infected with multiple organisms by direct spread. Staphylococcus aureus (*S. aureus*) is most of the time found in infections of bone that are hematogenous. Secondarily an inflammatory response that is of bony hyperemia is triggered by infecting agents. Bone demineralization is caused by hyperemia.[1]

The initial assessment of bone infection is blood testing radiographic imaging and microbiology of biopsy sample. Lucency is seen along the cement or metal-bone which is an important abnormal finding.[2] The most frequently used antibiotics for this treatment are Gentamycin, Tobramycin and Vancomycin.[3,4]

Vancomycin is used to treat Methicillin Resistant *Staphylococcus aureus* (MRSA) associated infection for decades. But because of its poor bone penetration, heteroresistance increase and glycopeptide tolerance search for newer agents has begun.[5]

One among them is curcumin that is seen in different animal models and human studies and was found to be safe even at very high doses (12 g/day). Curcumin is found to be effective against *Staphylococcus aureus* (*S. aureus*). It is a hydrophobic compound with low absorption and very poor solubility. Using some novel techniques like formulation of cubosomes, solubility of curcumin was enhanced.

Cubosomes is a bicontinous cubic liquid phase crystals. in which Monoolein: water, a binary system forms two types of cubic phase (Diamond and Gyroid), which are optically clear solid like phase at water content 20-40% (w/w) at room temperature. Bicontinous cubic liquid crystalline materials have gained lots of interest because of its unique structure that can be utilize to control drug release. To formulate such type of system amphiphilic polar molecules are utilized which form bicontinous water and oil channels. These are discrete, submicron, nanostructured particles of bicontinous cubic liquid crystalline phase, which are slightly spherical and range in size of 10 nm to 500 nm in diameter and appears like dots square shaped. They are produced

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Table 1: Different formulations of cubosomes from DoE.

Formulation code	Lipid (mg)	Surfactant (mg)
F1	250	50
F2	375	50
F3	500	50
F4	250	75
F5	375	75
F6	500	75
F7	250	100
F8	375	100
F9	500	100

by high energy dispersion of bulk cubic phase and stabilized using polymeric surfactants. The cubosomes were formulated by dilution method which is easy and adaptable enough to house any lipid and hydrotrope combination which on dilution forms cubic liquid crystalline material.[5]

Smart gels that are thermo sensitive and at physiological temperature undergo sol to gel transition have various applications in drug delivery. Both Pluronic F-68 and Pluronic F-127 are thermosensitive, biodegradable, and biocompatible surfactants, which are components that are Generally Recognized as Safe (GRAS). Hence Pluronic were utilized as vehicles for the ease of transport of the formulation along with providing a depot for sustained release of the drug, thus preventing the secondary operation for removal of the drug delivery system.[5]

MATERIALS AND METHODS

Materials

Pluronic F-127 and Pluronic F-68 were procured from Sigma life science, USA. Monoolein (GMO) was procured from Germany. curcumin, di Sodium EDTA. sodium bi carbonate, sodium lauryl sulphate and benzalkonium chloride were obtained from Loba Chemie Pvt, Ltd., India. Milli pore water was procured from Merck Ltd., India. Absolute alcohol was obtained from Changshu Fine Chemicals Co. Ltd., China.

Experimental design

A randomized, 3^2 factorial design was used to study the formulation of Cubosomes systematically. Two factors at three levels were taken and total nine experimental trials were conducted. Amount of monoolein (GMO) and Pluronic F-127 were taken as on the basis of trials taken independent variables at three levels (low, medium and high). Design-Expert 11.0 software (Stat-Ease Inc., USA) was used for generation and evaluation of the statistical experimental design.[6,7]

Formulation of Cubosomes

Cubic nanoparticles were prepared by hydrotrope dilution method. The desired amount of glyceryl monooleate (monoolein) taken in 10 mL volumetric flask and dissolved in 1 mL amount of alcohol (99.9%) and sheared for 30 sec following which the 5 mg of curcumin was added and vortexed for 2 min until clear yellow solution is formed (oil phase). 8 mL of Milli-Q deionized cold water containing the desired quantity of Pluronic F-127 (surfactant) previous vortexed for 1 min (water phase), was added gradually to the solution and stir continuously using vortex shaker under maximum speed for 5 min to achieve a homogenous formulation. The volume of the formulation was made up to the mark. After equilibration for 48 hr an optically isotropic cubic- phase gel was formed at room temperature.[8,9] The formulation chart for cubosomes was tabulated in Table 1.

Evaluation and Characterization of Cubosomes

Particle size and zeta potential

Using Photon Correlation Spectroscopy (PCS) and a zetasizer Nano ZS, the average particle size and Zeta Potential (ZP) were determined at 25°C (Malvern Instruments, UK). The refractive index was tuned at RI=1.456 in order to measure the particle sizes of lipid nanoparticle dispersions. A Mastersizer Hydro 2000MU was used to analyse the laser diffraction size[10] (Malvern Instrument, UK).

Entrapment Efficiency (EE)

The drug entrapment efficiency was determined by ultrafiltration. The upper chamber of a centrifuge tube was filled with 1 mL of cubosomes, and the tube was centrifuged for 10 min at 1000 rpm.[11] The following equation was used to compute the entrapment efficiency.

$$\%EE = \frac{\text{Total drug} - \text{free drug}}{\text{Total drug}} \times 100$$

Statistical analysis

Statistical analysis was done with SPSS (version 11.0, SPSS, Inc.). A p value of 0.05 or lower was regarded as statistically significant.[12]

Stability studies

Stability testing is done to confirm that the drug products are maintained within their limitations until the end of expiry. Stability studies were carried out on optimized formulation as per ICH guidelines and are subjected to different conditions. The stability studies were done for 3 months at 25°C.[13]

Fourier Transformed Infrared (FTIR) Spectroscopy

To determine the drug polymer interactions, FT-IR was used.[14] The ratio of the sample powder to KBr powder and drug was 1:3 and were prepared by applying 600 kg/cm^2 pressure. The spectral measurement was found on a FT-IR spectrophotometer

(Shimadzu, Model 8400, Japan), in a wave number region 4000 cm^{-1} to 400 cm^{-1} by powder diffuse reflectance.

Differential Scanning Calorimetry (DSC)

For both the pure medication and its preparations, DSC was performed. Spectral measurements were obtained on DSC-60 at a temperature range of 20-300°C.[15]

Identification of optimized formulation

By using design expert software (version 11.1.0, Stat ease Inc., Minneapolis, MN) the optimized formulation was found. As per the procedure given in previous table 1 the optimized formulation was prepared then it was incorporated into gel base to prepare a smart gel.[16,17]

Preparation of smart gel containing optimized cubosomes

The smart gel containing optimized cubosomes were prepared to increase the viscosity and improve the localization of the depot in the bone cavity. Pluronic-127 and Pluronic-68 served as gelling agents were directly dispersed in cubosomes aqueous dispersion. Smart gels of the cubosomes were prepared by cold method. The cubosomes based smart gels were prepared by adding Pluronic F-127 and Pluronic F-68 in the ratio 20:5 (% w/v) directly into the cubosomes dispersion and cooling the mixture overnight at 4°C. Direct addition of the polymers instead of a pre-formed smart gel prevents drugs dilution.[18,19]

Evaluation Parameters

Gelation temperature

The solution in a glass test tube was heated gradually in a temperature-controlled water bath while being vigorously shaken until it gelled.[20]

Gelation time

It was determined by technique known as tube inversion. In a thin-walled glass tube, 2 mL of smart gel was teken and immersed in a temperature-controlled water bath at the respective gelation temperature. At regular intervals the test tube was observed. On the basis of flow or no-flow criteria the gelation time was determined by using test tube inversion technique and the time was noted for the whole system.[21]

Syringeability test

It was done using the procedure outlined by Schuetz *et al.* The syringeability time was determined to be the typical amount of time required to eject 5 mL of formulation under constant pressure (0.5 kg mass).[20]

Viscosity

The formulated cubosomes incorporated smart gels were measured for viscosity by using Brookfield viscometer with spindle No. 5 at a speed of 50 rpm.[21]

Physiochemical characterisation: Organoleptic evaluation

Comparison of F7 responses with predicted values obtained by DoE was done.

Phase separation and drug precipitation

A 5 mL optimized formulation of cubosomes was stored for 3 months at ambient temperature 25°C /60% RH in stability chambers and visually observed for phase separation and drug precipitation. Optimized formulation was visually examined on weekly basis for 3 months.[22]

Creaming

F7 was analyzed for creaming. The dispersion formed was assessed visually for creaming during the storage time at 25°C/60% RH for 3 months.[22]

Discoloration

To identify the discoloration (change in colour), sample was inspected visually. Optimized formulation was checked for change in colour after 1 week of preparation, and continued for next three months.[23]

Physical stability of optimized formulation

Physical stability of cubosomes of curcumin (formulation F7) was evaluated at 25°C/60% RH maintained by stability chamber for three months.[23]

In vitro release study

This study was performed for pure curcumin and optimised formulation (F7) using dialysis bag technique. pH 1.2 containing 1% w/v BKC and pH 6.8 phosphate buffer containing 0.5% w/v SLS were used dissolution media. Free drug was released into the dissolution media and cubosomes were held by the dialysis bag. The bag which was using for the study was washed using distilled water. 2 mL of formulation was placed in dialysis bag maintained the temperature at 37±0.5°C and stirred the solution at 50 rpm. At fixed time intervals the samples were withdrawn and to maintain sink conditions, the fresh dissolution medium was replaced. Samples withdrawn were analysed for the drug content by UV-visible spectroscopy.[24]

RESULTS

Particle size and zeta potential of Cubosomes

The mean diameter of formulation F7 was around 186.27nm. The zeta potential of the optimised formulation F7 was found to

Table 2: DoE responses of the different cubosomes formulation.

Formulations No.	Factor 1 A: Lipid (mg)	Factor 2 B: Surfactant (mg)	Response 1 Particle size (nm)	Response 2 Entrapment efficiency (%)
F1	250	50	219.45	67.02
F2	375	50	230.56	65.87
F3	500	50	302.48	35.66
F4	250	75	195.47	70.11
F5	375	75	251.15	52.67
F6	500	75	286.12	38.76
F7	250	100	186.27	71.24
F8	375	100	262.34	45.96
F9	500	100	279.11	41.55

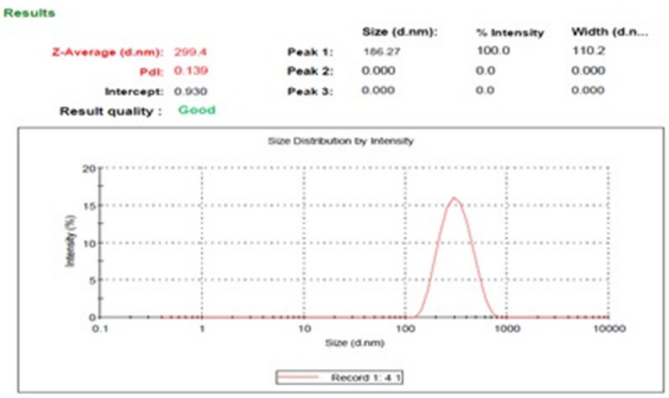

Figure 1: Particle distribution and zeta potential values of formulation F7.

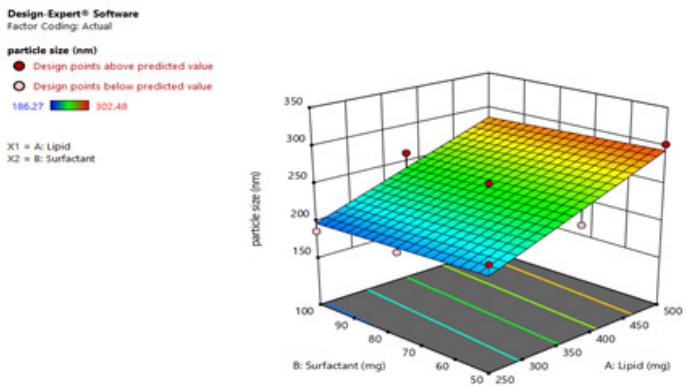

Figure 2: 3D graph of particle size.

be -17.5 mV which indicates the particles are moderatey stable (Figure 1). The responses to particle size were shown in Table 2.

Drug entrapment efficiency

Optimised cubosomes formulation EE was found to be 71.24%. It states major encapsulation of drug is satisfactory. The relative low encapsulation efficiency for hydrophilic drugs was ascribed to the limited entrapping capacity of the water channel in the formulated cubosomes (Table 2).

ANOVA responses

Response 1 (R1): Particle size

ANOVA responses for particle size were shown significantly and the results were depicted in Table 3.

The Predicted R^2 of 0.7687 is in rational arrangement with the Adjusted R^2 of 0.8680; i.e. the difference is less than 0.2. A ratio greater than 4 is desirable and in this case obtained ratio is 11.371 that indicates an adequate signal. 3D graph for particle size was showed in Figure 2. Final equation in terms of coded factors: Particle size: +245.88+ 44.42 A - 4.13 B.

This response surface plot (3D) indicates the effect of X1(lipid) and X2 (surfactant) on Y1(particle size).

Response 2: Entrapment efficiency

ANOVA responses for particle size were showed significant and the results were depicted in Table 4.

The Predicted R^2 of 0.6929 is in rational arrangement with the Adjusted R^2 of 0.8250; i.e. the difference is less than 0.2. A ratio greater than 4 is desirable and in this case obtained ratio is 9.804 which indicates an adequate signal (Figure 3).

Final Equation in Terms of Coded Factors

Entrapment efficiency = +54.32 – 15.40 A - 1.63 B

Response surface plot (3D) indicates the effect of X1(lipid) and X2(surfactant) on Y2 (entrapment efficiency). And from the above results the desirability was found 0.903 (Figure 4) with lipid and surfactant.

Table 3: ANOVA for response surface linear model (R1).

Source	Sum of squares	df	Mean squares	*F*-value	*p*-value	Remarks
Model	11941.08	2	5970.54	27.30	0.0010	Significant
A-Lipid	11838.82	1	11838.82	54.12	0.0003	
B-Surfactant	102.26	1	102.26	0.4675	0.5197	
Residual	1312.45	6	218.74			
Cor Total	13253.52	8				

Table 4: ANOVA for response surface linear equation (for entrapment efficiency).

Source	Sum of squares	df	Mean squares	*F*-value	*p*-value	Remarks
Model	1438.97	2	719.48	19.86	0.0023	Significant
A-Lipid	1422.96	1	1422.96	39.28	0.0008	
B-Surfactant	16.01	1	16.01	0.4419	0.5309	
Residual	217.33	6	36.22			
Cor Total	1656.30	8				

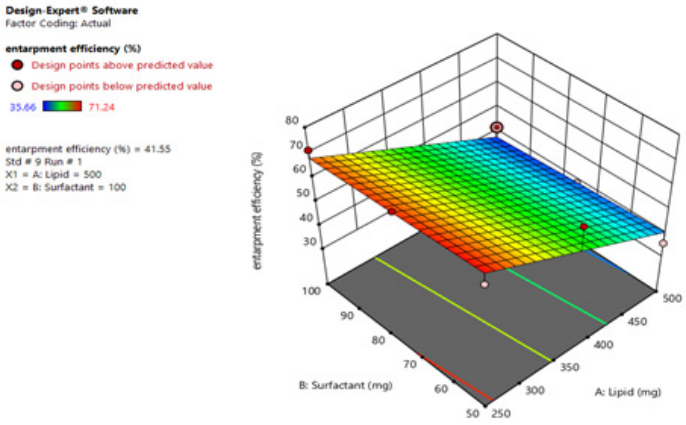

Figure 3: 3D Graph of entrapment efficiency.

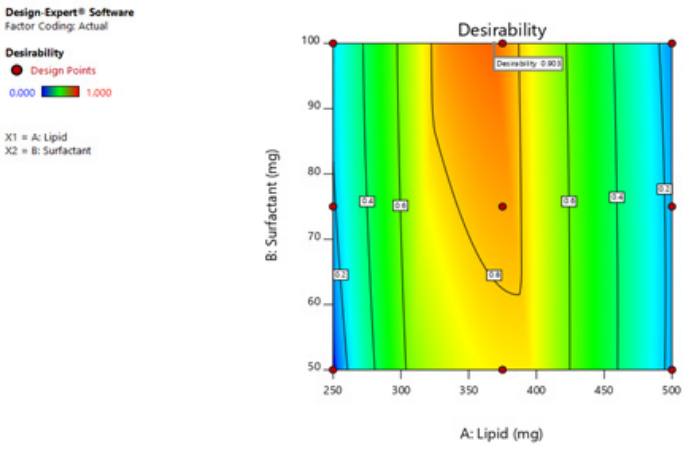

Figure 4: Desirability of the cubosomes formulation.

Evaluation and Characterisation tests for cubosomes loaded smart gel

Gelation temperature

Prepared cubosomes loaded smart gel has shown dependent reversible sol to gel transition. This smart gel system changed from sol to gel at 34±1°C, which is below the body's temperature.

Gelation time

To maintain the smart gel at the injection site and allow for a prolonged localised drug release, an instant shift from sol to gel is necessary. According to the results, the produced cubosomes filled with smart gel react quickly to changes in gelation temperature in less than 58±1 sec.

Syringeability

Time recorded was found to be 11±1 sec. The result showed that the cubosomes loaded smart gel could be easily syringed through an 18-gauge needle.

Viscosity

Viscosity is a rheological parameter and is very important for any thermosensitive gel systems, which is mainly involved in its application and *in vivo* performance. The cubosomes loaded smart gel formulation showed a viscosity of 2242±3 cps and 65176±20 cps at 5±3°C and at 37±3°C respectively where significant change can be seen. This can be attributed to the thermo sensitivity of Pluronic which causes to a sol to gel conversion at various temperature. Results showed in Table 5.

Table 5: Evaluation parameters of smart gel Optimized formulation (F7).

Sl. No.	Evaluation parameters	Results
1	Gelation temperature	34±1°C
2	Gelation time	<58±1 sec
3	Syringeability	11±1 sec
4	Viscosity	2242±3 cps and 65176±20 cps at 5±3°C and at 37±3°C

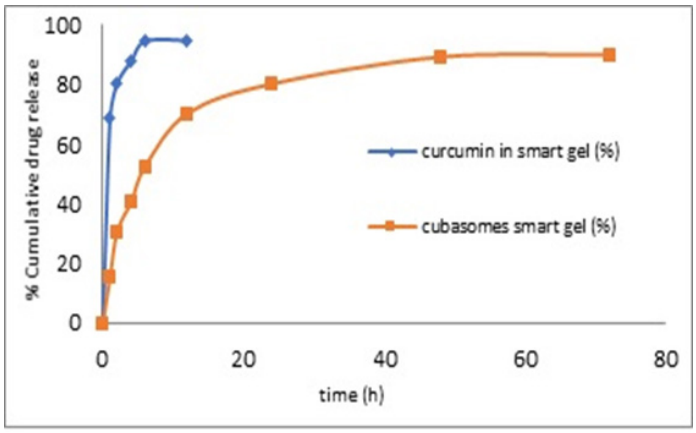

Figure 5: *In vitro* drug release for curcumin loaded smart gel and cubosomes loaded smart gel.

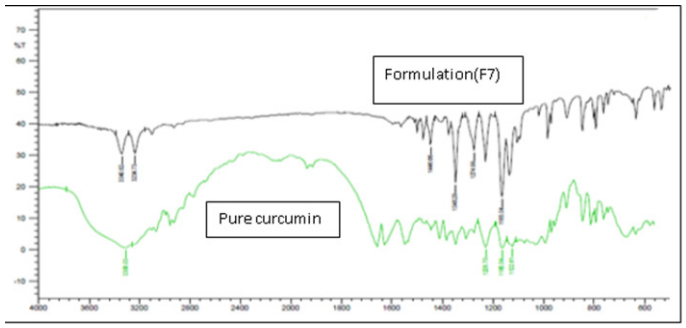

Figure 6: FTIR spectra of pure curcumin and optimized formulation F7.

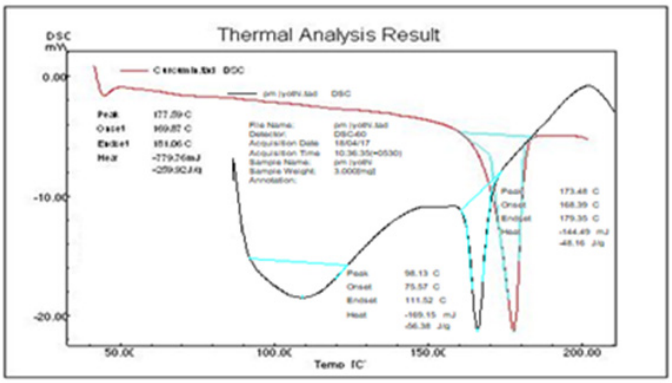

Figure 7: DSC thermograms of pure curcumin and optimized formulation.

In vitro drug release studies

A quick and almost complete release was observed from curcumin loaded smart gel after 6 hr when compared to cubosomes loaded smart gel which is shown in Figure 5. Biphasic release profile of cubosomes loaded smart gel was found. Initial release was around 52.6 ± 0.03% of drug during the first 6 hr, followed by a moderately slow release of the remaining drug for a period of 72 hr. The higher initial release is mainly due to the adsorbed drug or weakly bound drug to the relatively larger surface of cubosomes.

FT-IR studies

Figure 6 illustrates the FT-IR spectra of pure curcumin and along with its formulation. The FT-IR spectrum of curcumin showed Phenolic OH group at 3511 cm^{-1}, C=O vibrations at 1635 cm^{-1}, C-H stretching at 1351 cm^{-1}, O-H Stretch primary Alcohol at 3368 cm^{-1}, O-H Stretch intracavity H_2O at 3532 cm^{-1} and O-H Stretch at 83280 cm^{-1}. All the characteristic peaks of curcumin along with minor shifts were shown by spectra of the optimized formulation. The FT-IR spectrum of optimized formulation was showed C Phenolic OH group at 3513 cm^{-1}, C=O vibrations at 1630 cm^{-1}, C-H stretching at 1348 cm^{-1}, O-H Stretch primary Alcohol at 3310 cm^{-1}, O-H Stretch intracavity H_2O at 3530 cm^{-1} and O-H Stretch at 3244 cm^{-1}. These data evidently indicate that

Table 6: Physical stability study results of formulation (F7) loaded smart gel.

Parameters	1st month		2nd month		3rd month	
	Weeks		Weeks		Weeks	
	2nd	4th	2nd	4th	2nd	4th
Phase separation	-	-	-	-	+	+
Discoloration	-	-	-	-	-	-
Creaming	-	-	-	-	-	-

+++ are unacceptable; ++ are significant changes; + are acceptable; – is no change.

the characteristic absorption peaks of curcumin were seen in optimized formulation, and it indicates that drug and optimized formulation had no interaction between them.

DSC studies

The DSC of the optimised formulation F7 showed a decrease in the drug's melting point, which may be related to a decrease in the crystallinity of the compound, so deformation of peaks was visible as in Figure 7. The thermogram of pure drug has showed a sharp endothermic peak at 177.5°C, which corresponds to its melting point.

Stability study of optimized formulation

Creaming, discoloration and phase separation of product was done for optimized formulation. Biweekly basis visual assessment of optimized formulation loaded in smart gel was observed for 3 months at storage condition 25°C/60% RH and the results obtained were depicted in Table 6.

Visual assessment of optimized formulation didn't show any sign of phase separation and drug precipitation. During visual assessment no sign of creaming and discoloration occurred. 2nd and 4th week of third month showed minor changes in phases. But these changes were within acceptable ranges, upon shaking dispersion was uniformly dispersed. Such an observation was expected because GMO was dispersed in the presence of poloxamer 407 which provide adequate colloidal stability to the dispersion by adsorbing at the surface of dispersed particles. GMO and Poloxamer 407 ratio 5:2 (250 mg: 100 mg) were adequate to provide stearic stability by preventing the coalescence of dispersed particles resulted in uniform distribution of dispersed phase. Due to these effects optimized formulation didn't show any significant sign of phase separation and creaming during the storage self-life of three months. yellowish colour (imparted by curcumin) of optimized formulation was maintained throughout the study didn't show any discoloration. That indicated no change in product.

CONCLUSION

An attempt to formulate a cubosomes containing curcumin was formulated by hydrotropic dilution technique using pluronic F-127, ethanol, glyceryl monooleate and water. The cubosomes optimization was achieved by factorial design. The optimized formulation was incorporated into the smart gel and characterization tests were performed. The formulated smart gel containing the optimized cubosomes formulation (F7) when evaluated showed good rheological properties, release characteristics and stability studies. Hence it might be ideal formulation for the treatment of Osteomyelitis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

FTIR: Fourier-transform infrared spectroscopy; **DSC:** Differential Scanning Colorimetry; **ICH:** International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use; **DoE:** Design of Experiments; **rpm:** Rotation per minutes.

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