FABRICATION OF SERRATIOPEPTIDASE LOADED CUBOSOMAL GEL FOR TRANSDERMAL DRUG DELIVERY

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Abstract

Transdermal drug delivery (TDD) is a painless method of delivering drug systemically by applying a drug formulation onto intact and healthy skin. Serratiopeptidase is a proteolytic enzyme which shows effective treatment for inflammation, pain and has been used for various treatment namely arthritis, atherosclerosis, post - operative swelling and bronchitis, sinusitis, fibrocystic breast disease. Cubosomes are increasingly being recognized and utilized as a drug delivery system, particularly in the treatment of diseases like cancer. Topical gel formulation of serratiopeptidase was prepared using polymer such as pluronic F- 127 and lipid such as a Glyceryl monooleate. The formulation was optimized using 3^2 full factorial design and was evaluated for various parameters such as particle size, polydispersity index, zeta potential, invitro drug release and surface morphology was analysed by transmission electron microscopy. The optimized cubosomal formulation was incorporated into a gel and was evaluated for various parameters such as pH, spreadability, washability, viscosity, drug content, and in-vitro drug diffusion with franz diffusion cell. The optimized formulation F1 cubosomal formulation showed an uniform and homogenous spherical shaped structure as confirmed by TEM imaging, the particle size of the compound was between 200-500nm, it showed Entrapment efficiency 87.3%, polydispersity index was 0.123cps, and zeta potential was 87.73%. The optimized gel showed better results due to its small particles size and high entrapment efficiency leading to its better topical delivery of serratiopeptidase cubosomal gel in arthritis.

Keywords: Transdermal drug delivery, Serratiopeptidase, Cubosomes, Entrapment efficiency, TEM analysis.

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Introduction

Transdermal drug delivery system is considered as self-containing, discrete dosage form which upon application to the intact skin, delivers the drug across the skin at a controlled rate into the systemic circulation by a process of diffusion through which the drug enters into the bloodstream thus providing a enhanced therapeutic effect [1].The flow of the drug within the bloodstream can be ensured into a continuous flow due its high concentration within the blood stream, thus undergoing the diffusion process continuously in order to maintain the level of drug in the systemic circulation. Transdermal drug delivery system gives an scope of an improved therapeutic safety and efficacy by providing the release of drug at a predetermined and controlled rate [2].

Serratiopeptidase (serratia E-15 protease) is a proteolytic enzyme also known as serralysin, serrapeptase, serratia protease .This enzyme has shown effective treatment for inflammation , pain and has been used for various treatment namely arthritis, atherosclerosis ,post-operative swelling and pain, bronchitis, sinusitis, fibrocystic breast disease. Serratiopeptidase was originated from microorganism genus Serratia species E-15 which are non-pathogenic enterobacteria. This microorganism was originally separated from the intestine of a silkworm Bombyxmori .Serratiopeptidase is ametalloprotease containing zinc atom which has its importance in proteolytic activity of enzyme and has molecular weight of 45-60 kDa. 10ml of serratiopeptidase equals to 20,000 units of enzyme activity [3,4].

Cubosomes are nanoparticles that are formed by self-assembling of lipids in presence of water which results in the formation of 3-dimensional nanostructure called cubic phase. The cubic phase contains water channels which are enclosed by curved bilayer of surfactant molecules.

This surfactant layer can dissolve both oil and water, forms a saddle like structure and extends into three dimensions. These nanoparticles are used as active transport vesicles [5].

Cubosomes are generally prepared by top down technique, bottom up technique, emulsification and fabrication. The formation of Cubosomes involves mixing of suitable lipid and polymer/stabilizing agent using these various techniques. The formation relies on balanced amount of lipid, polymer and water. The particle size of Cubosomes depends on the lipid used and preparation method adopted. The size range for Cubosomes is generally between 100 to 500 nm. The technique employed here is top down technique and the lipid used here is Glyceryl Monooleate (GMO) [6-8].

Transdermal drug delivery is an innovative and more feasible option for the delivery of drug intended for topical use. Moreover, the transdermal drug delivery offers an increased patient compliance. Serratiopeptidase is used to treat pain, inflammation and diseases like arthritis, carpal tunnel syndrome, sinusitis among many others. Serratiopeptidase is a form of proteolytic enzyme consisting of peptides that is hydrophilic in nature and can undergo enzymatic degradation in gastrointestinal tract (GIT). Although upon oral administration the drug can get easily absorbed in intestine but its high vulnerability towards gastric degradation hinders its use as a potential drug for oral administration. To overcome this limitations, an alternative method of Transdermal drug delivery system is adopted as an ideal candidate to transfer the drug as topical solution of cubosomal gel for arthritis. Application of this nanoparticle cubosomal gel on skin would be more appropriate method to transfer the drug into a desired area.

The findings from earlier literature review have anticipated the need of the current study.

Following is some of the literature review of already existing studies.

Salwa Salha along with his colleagues conducted a research to create targeted pain relief and joint stiffness using transdermal cubosomes loaded with etodolac. The intention was to achieve controlled and sustained release of etodolac via noninvasive skin application. By employing emulsification and homogenization methods, the researchers experimented with different ratios of poloxamer 407 and monoolein to formulate the cubosomes. The outcomes exhibited small particle sizes and a high rate of drug encapsulation. Controlled drug release was observed with varying release rates during in-vitro studies. The cubosomes assumed cubic and hexagonal shapes while effectively encapsulating the drug in an amorphous state. Conducting skin penetration studies, the researchers noted rapid drug penetration followed by a slower release over a 24-hour period. When pharmacokinetic analysis was performed on human volunteers, the results indicated increased bioavailability, a longer half-life, and a higher mean residence time in comparison to oral capsules. Overall, the etodolac-loaded cubosomes demonstrate considerable potential as a transdermal system for arthritis treatment [9].

In a studies performed by Ryan Varghese et al, in his review on Cubosomes in cancer drug delivery elaborated on nanovesicluar drug delivery system. Cubosomes have gained significant recognition and application as a drug delivery system, specifically in addressing conditions like cancer.

These versatile carriers exhibit great potential in both therapeutic and diagnostic functions (theranostics) and offer multiple administration routes, including oral, topical, and intravenous methods. This assessment seeks to underscore the recent advancements and hurdles faced in the utilization of cubosomes for diverse cancer types. Additionally, it discusses the necessary steps to establish cubosomes as a promising nanotechnological intervention for cancer treatment [10].

In another study by Hoda E Teba and his colleagues on a novel cubosomal delivery system for ocular drug delivery system of acetazolamide which has proven to be a successful treatment for glaucoma. However, its current form as systemic tablets has associated systemic side effects, and its application as a topical treatment is hindered by its limited solubility and low corneal permeability. To address these issues, the study focused on using cubosomes, a specialized drug delivery system known for its advantages, such as a large surface area, thermal stability, and the capability to encapsulate various molecules. Specifically, acetazolamide-loaded cubosomes were developed as eye drops for glaucoma treatment [11].

In a systemic review by Shivani Bhagat et al on Serratiopeptidase, that discussed about the efficacy and safety of Serratiopeptidase in clinical settings, employing a systematic assessment of previously published articles. Comprehensive searches across various databases and additional sources yielded 24 studies that met the predefined criteria for inclusion. The quality of these studies was rigorously assessed according to established guidelines. The findings suggest that Serratiopeptidase exhibits promise as both an anti-inflammatory and analgesic agent. However, it is important to acknowledge that the studies encountered methodological limitations and lacked sufficient safety and tolerability data. Therefore, further research is imperative to furnish evidence-based recommendations regarding the use of Serratiopeptidase for analgesic and antiatherosclerotic purposes, as well as to ascertain its long-term safety profile [12].

A research work performed at Dr. B.R. Ambedkar National Institute of Technology, Jalandhar, India, by Sandeep Kumar and his associates, on formulation and characterisation of cubosomal nanoformulation of Serratiopeptidase This research has presented a study on the application of magnetic nanoparticles (MNPs) coated with serratiopeptidase enzyme for targeted treatment. To achieve this, the researchers immobilized the enzyme on the MNPs using covalent bonds through glutaraldehyde after amino functionalization of the nanoparticles. Several parameters were examined during this process. The resulting enzyme-bound MNPs, called EMNPs, underwent analysis for size, crystallographic identity, phase purity, zeta potential, magnetic properties, as well as elemental and thermal properties [13].

Materials and Methods

Materials

Serratiopeptidase enzyme was procured from Advanced Enzyme Technologies Ltd. (Thane, Maharashtra, India). Mohini organics Pvt. Ltd. Provided Glyceryl Monooleate. Pluronic F-127 was acquired from Sigma Life Science Pvt. Ltd. Potassium dihydrogen orthophosphate purified (99-101%) and Di-sodium hydrogen orthophosphate purified (98.6%) was obtained from SD fine-Chem Ltd. (Mumbai, India). Sodium Chloride (99.5%) was purchased from Loba chemie Pvt. Ltd. H.P.M.C K 4 M was purchased from Yarrow Chem Products (Mumbai, India).

Methodology

Preformulation studies of Serratiopeptidase

Determination of Absorption Maxima and Standard Calibration curve

The wavelength of the maximum absorbance was recorded. Accurately weighed 10 mg of serratiopeptidase was dissolved in 100 mL of phosphate buffer 7.4pH. An absorption maximum was determined using this solution at wavelength of range 800 to 200nm. Calibration curve was determined using concentration of 100, 200, 300, 400 and 500µg/mL at wavelength 277nm [14,15].

Preparation of Serratiopeptidase loaded Cubosomes

The Cubosomes suspension was prepared using a technique called the Top-down Technique. Initially, the temperature was maintained at 40ºC, and the lipid (GMO) was melted using a heating magnetic stirrer. The active pharmaceutical ingredient (API) was then added up to the melted lipid, and the mixture was stirred and mixed up using a magnetic bead. Simultaneously, the polymer was melted in a separate beaker. Various concentrations of lipid and polymer are used for formulation. The Lipid-Drug mixture was combined with the polymer and thoroughly mixed for a few minutes in varying concentrations as shown in table 1.

Further water maintained at the same temperature was added drop by drop to the above mixture using a syringe, while maintaining a constant stirring speed of 700 rpm. Once cooled, the cubosomes were homogenized by subjecting the suspension to a stirring speed of 15000 rpm for 15 minutes using an IKA T 18 digital Ultra Turrax. Subsequently, the

cubosomal suspension was sonicated for 10 minutes using an Ultrasonic Probe Sonicator VC750, while keeping it under cold temperature conditions. Finally, the formed cubosomal suspension was stored in an ambercolored bottle in the refrigerator [16-18].

Table 1 Formulation table 3^2 Full factorial design

Formulation code	Drug (mg)	Polymer % (w/v)	Lipid % (w/v)
F1	10	0.5	1
F2	10	0.5	2
F3	10	0.5	3
F4	10	1	1
F5	10	1	2
F6	10	1	3
F7	10	2	1
F8	10	2	2
F9	10	2	3

Characterization of Serrtiopeptidase Cubosomes

Estimation of Particle Size (Vesicle Size), Polydispersity Index (PDI) and Zeta Potential for the formulated Serratiopeptidase Loaded Cubosomes

The average particle size of the Cubosomes, their size distribution (polydispersity index; PDI) and their zeta potential were measured by Zetasizer Lab Malvern Instruments Ltd., UK which works on the principle of dynamic light scattering at an angle of 90°C for measuring particle size and PDI [19]. The principle for measuring zeta potential is mixed-mode measurement phase analysis light scattering.

Estimation of entrapment efficiency (% EE) for cubosomal suspension

The formulated cubosomes suspension was centrifuged at 15000 rpm (rotation per minute) and 4°C for 15 min using High speed Refrigerated centrifuge Kubota Model 7000/230V. The supernatant liquid was extracted and collected to determine the non-entrapped or the free serratiopeptidase drug by spectrophotometric analysis (Shimadzu UV-1900 spectrophotometer) at 277nm. To determine the percentage entrapment efficiency of Serratiopeptidase in the formulated Cubosomes was evaluated by determining the amount of entrapped and free drug and subtracting the amount of free serratiopeptidase from the total amount of serratiopeptidase used to prepare the Cubosomes [20].

The entrapment efficiency (E.E %) was estimated according to the following equation:

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

Optimization of Formulation using DOE software

A 3^2 full factorial design was used to determine the optimized formulation with optimum concentration of polymer and the lipid. The independent variables selected were concentration of polymer and lipid whereas the dependent variables selected were particle size, entrapment efficiency, PDI, Zeta potential as depicted in table 2. Optimised formulation was used for further studies and gel preparation [21].

Table 2 Coded values for optimization of cubosomal formulation

		Coded Values		
	Low (-1)		Medium (0)	High (+1)
		Independent Variables		
Polymer Concentration % (w/v)	0.5		1	2
Lipid Concentration % (w/v)	1		2	3
		Dependent Variables		
Particle Size (nm)	Minimum			
Entrapment Efficiency (%)	Maximum			

Transmission electron microscopy (TEM) analysis

The optimized formulation of Cubosomes was evaluated for morphological surface characterization by using Transmission electron microscopy (TEM) analysis [22].

In-vitro drug release study

The dialysis bag method was used to determine the in-vitro drug release profile of cubosome dispersion. Dialysis bags were cleaned and soak in deionized water. The tubing-based dialysis bag was placed into a beaker with 90mL of the phosphate buffer solution of pH 7.4 after the cubosomal dispersion was pipetted into the bag and sealed. The vessel was set over a magnetic stirrer running at 50 revolutions per minute, and a temperature of 37°C ± 0.5°C was maintained. To keep the sink condition throughout study, samples were collected at predefined time periods and instantly replaced with a similar fresh media. Samples were diluted and estimate for drug content by utilizing UV/visible spectroscopy at 277nm [23].

Preparation of Serratiopeptidase loaded Cubosomal gel

Take weighed quantity (10g) of cubosomal suspension in a beaker. Keep it on magnetic stirrer. Add 300mg HPMC K4M pinch by pinch to the suspension and stir using magnetic bead at 500 rpm till viscous gel is formed [24].

Evaluation tests for Serratiopeptidase Cubosomal gel

The prepared gel was evaluated for different parameters like pH, spreadability, Viscosity, Drug Content and in-vitro drug release [25].

Determination of pH value

The pH of gel was estimated by using a digital pH meter. 1g of serratiopeptidase gel was mixed in distilled water till a uniform suspension is formed and pH of solution was measured [26].

Determination of spreadability

1g gel was stationed in between 2 glass slides and a weight of 20gm was kept on slides for 5 minutes to compress the gel to a uniform thickness. The time in seconds and the length in diameter the gel spreads between the two slides was taken as a measure of Spreadability [27]. It is calculated using formula

$S = M*L/t$

Where, S= Spreadability, M= weight kept on upper slide, L= length, t= Time taken in seconds

Determination of viscosity

To determine the rheological properties of the formulated gel viscosity is determined by using Brookfield Cap 2000+ Viscometer (Dial type). For viscosity measurement, spindle no. 2 is used, for fixed time of 2mins at 100 rpm [28].

Drug content 1g of gel was dissolved in 100 mL solvent (phosphate buffer 7.4pH). From that 1mL was taken and diluted to 10mL. Then analyse the drug content with the Shimadzu UV-1900 spectrophotometer at wavelength of 277nm [29].

In vitro drug diffusion study

Small strip of dialysis membrane was immersed and kept in phosphate buffer 7.4pH overnight. All formulations and optimized gel were subjected to in-vitro diffusion. Franz diffusion Cell was used for the study, the receptor compartment was filled with phosphate buffer 7.4pH and kept at temperature 37+0.5°C and stirred with magnetic stirrer. About 3g of gel was placed on the cellophane membrane. 1 mL of sample was withdrawn from the receptor compartment at time intervals of 30mins, 1.5hrs, 2hrs, 4hrs and 6hrs and replaced with same volume of medium. All samples were diluted using phosphate buffer to 10mL and analysed for serratiopeptidase drug content using Shimadzu UV-1900 spectrophotometer at wavelength 277nm [30-32].

Result and Discussion

Determination of Absorption Maxima and Standard Calibration curve

The wavelength at which highest absorption was observed was at 277 nm. The standard calibration curve was created by plotting concentration vs absorbance (Figure 2). The standard calibration curve was linear at 277 nm in beer's range and the equation was y=0.001x - 0.010.

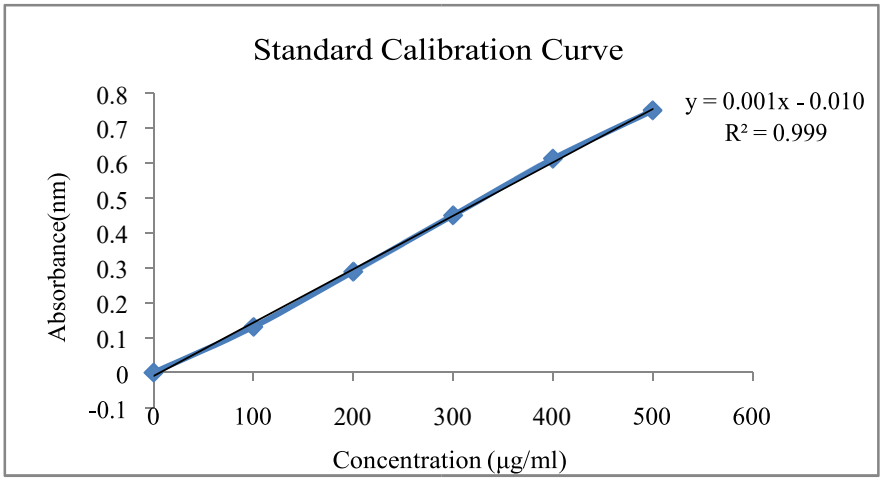

Fig. 1 Standard calibration Curve

Characterization of Serratiopeptidase loaded Cubosomes

Particle Size (Vesicle size), Zeta Potential of Cubosomes and Entrapment efficiency

Particle Size and Zeta Potential of Cubosomes were performed by dynamic light scattering technique by using Zetasizer lab Malvern instrument Ltd.

The cubosomes diameter was found to be in range of 100 to 743nm and the average particle size was 353.19nm.

The Entrapment Efficiency of cubosomal formulations was found to be in range of between 12.2% to 95.26%.F1 was selected as optimized product based on its small size, low PDI and high entrapment efficiency. The results of characterization parameters were recorded in table 3 and Figure 3 and Figure 4 shows the particle size and zeta potential graphs respectively.

Table 3 Characterization parameters of Serratiopeptidase loaded Cubosomes

Formulation	Particle size (nm)	Polydispersity index (PDI)	Zeta Potential (mV)	Entrapment efficiency (%)
F1	200.9	0.4837	-52.8	87.73
F2	225.4	0.5152	-52.8	88.2
F3	347.3	1	-51.91	83.73
F4	374.6	1	-52.8	56.53
F5	276.8	1	-56.93	70.93
F6	104.8	1	-32.45	12.2
F7	123.8	1	-42.21	28.46
F8	135.4	1	-35.21	16.84
F9	408.8	0.1239	-52.8	95.26

Optimization of Formulation Variables

Effect of Pluronic f-127 on formulation of Cubosomes

If the concentration of Pluronic f-127 increased then there will be decrease in entrapment efficiency and drug release from Cubosomes. The optimum concentration of Pluronic f-127 for cubosomes formulation was found to be 0.25% and it showed the entrapment efficiency of 87.73%.

Effect of Glyceryl Monooleate (GMO) Concentration on Cubosomal formation

Cubosomes were acquired using GMO Concentration between the ranges of 1% to 4.5%. Cubosomes having GMO concentration of 1% was optimized and entrapment efficiency observed was 87.73%. The counter plots pertaining to effect of entrapment efficiency and particle size are shown in Figure 5 and 6 respectively.

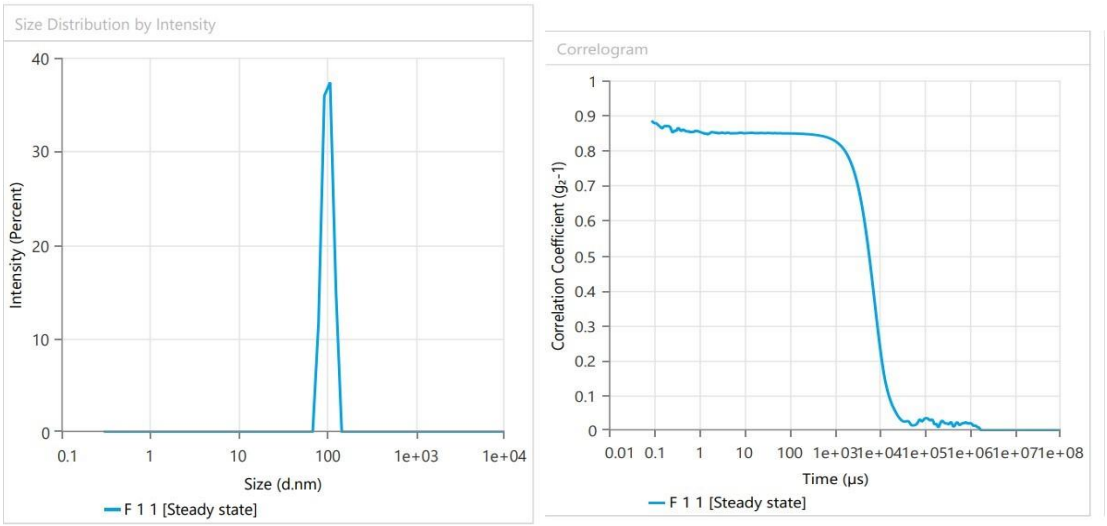

Fig. 2 Particle Size of Optimized Formulation

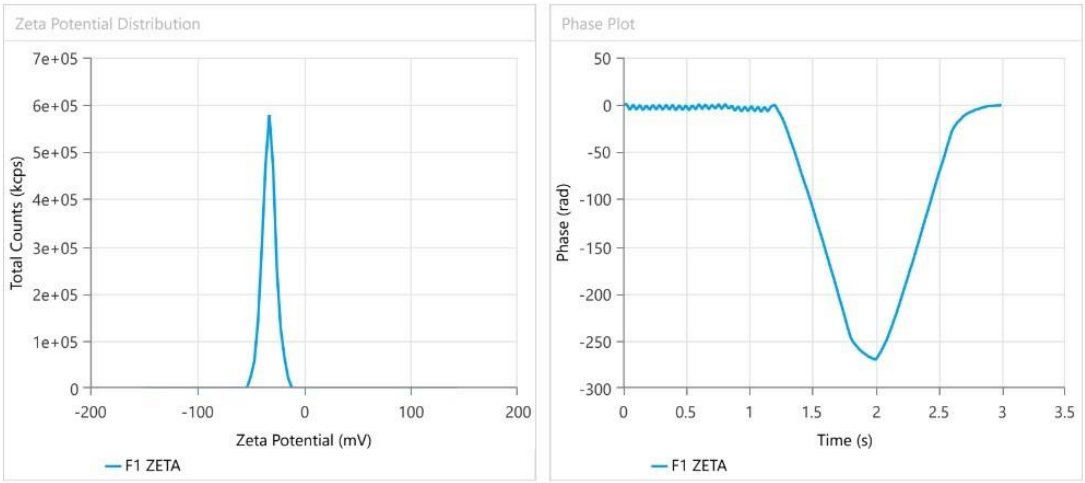

Fig. 3 Zeta potential of Optimized formulation

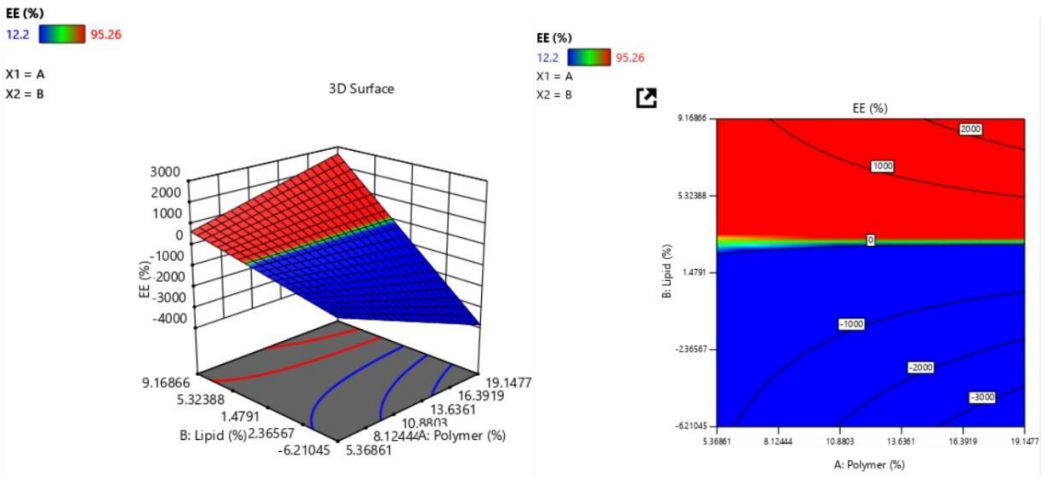

Fig. 4 Contour plot showing effect of lipid and polymer on Entrapment efficiency

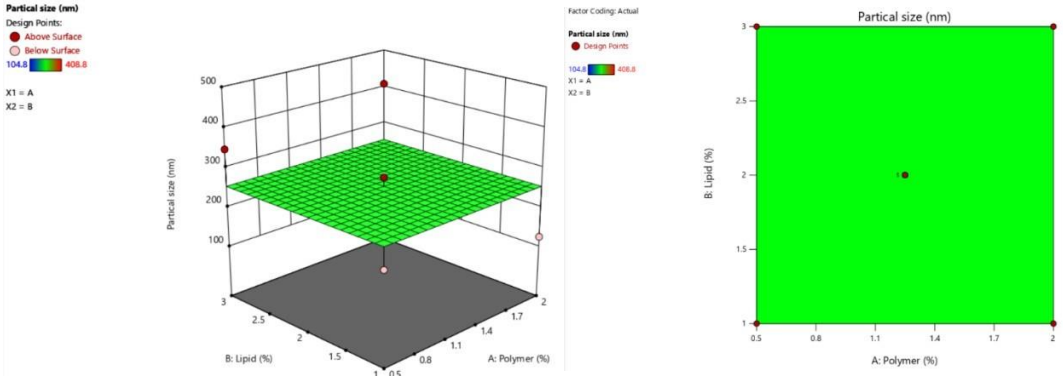

Fig. 5 Contour plot showing effect of lipid and polymer on particle Size

Surface Morphology of Cubosomes

The Surface morphology of the optimized cubosomal formulation was studied with TEM analysis. The formulation showed a uniform and homogenous spherical with particles size in nano ranges (Figure 7).

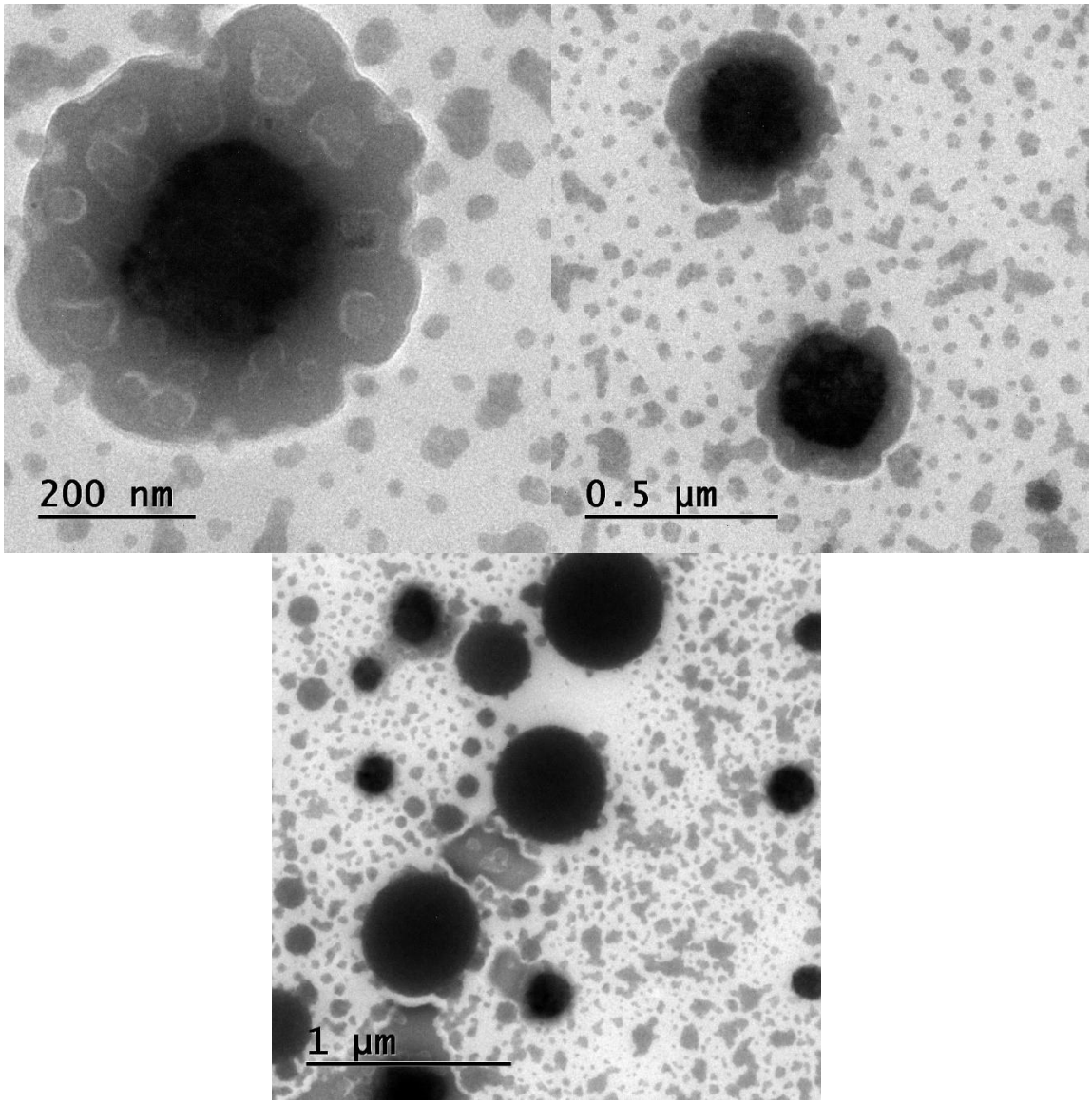

Fig.6 TEM Analysis Images of Serratiopeptidase loaded Cubosomes

In-vitro drug release study

The graph of the cumulative percentage of the drug released over time was plotted to obtain the in-vitro drug release profiles of Serratiopeptidase from the optimized batch F1 at pH 7.4. In order to dissolve the released drug during the release studies, PBS (pH7.4) was supplemented with 0.1 percent (v/v) sodium lauryl sulphate. 0.1 percent (v/v) of sodium

lauryl sulphate was added to the release medium PBS (pH 7.4). According to the figure shown, the drug release from the optimized F1 batch with an initial burst release of approximately 23.45% respectively in 1hr, followed by maximum amount of drug released was observed up to 24hrs. It indicates that the formulated cubosomes shows sustained prolonged drug release. 81.68% of the drug is released from thee formulated Cubosomes after 24hours (Figure 8).

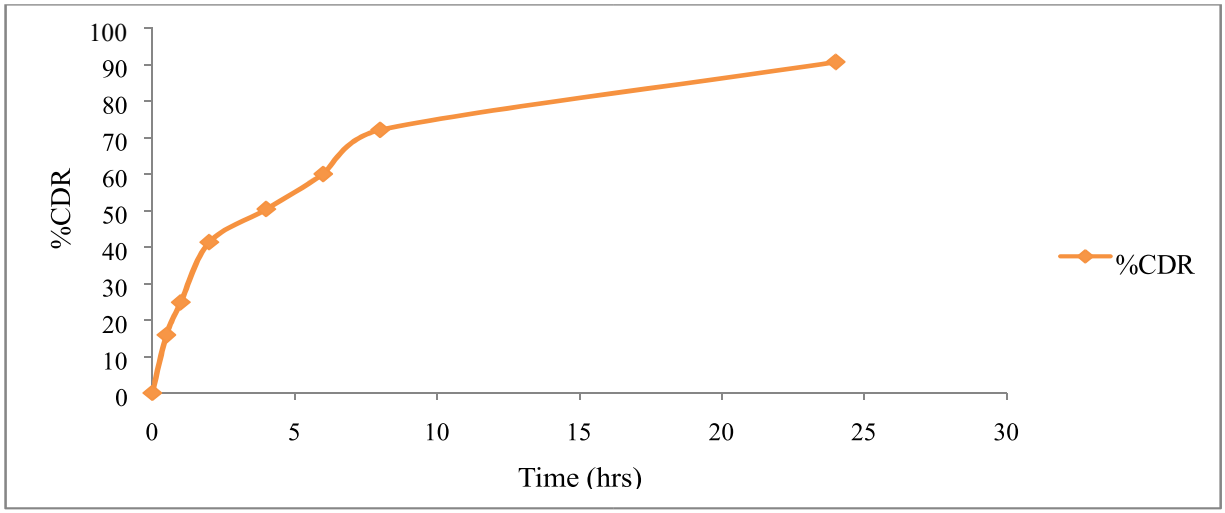

Fig. 7 Drug release profile of the formulation

In-Vitro Diffusion study

The estimate the drug release profile of the drug from the formulated cubosomes the in-vitro studies were performed for all the formulations. The % Cumulative drug diffusion from the formulation was recorded in table 4 and figure 9. The optimized formulation F1 showed maximum drug diffusion of 68.82% after 6 hours when compared to other formulations. It can be noted that all the formulations showed an initial burst of release of the drug from formulation followed by a sustained release for 6 hours.

Table 4 Determination of drug diffusion study for all formulations

Time	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.5	11.23	12.17	6.55	8.42	6.55	12.17	7.49	11.23	7.49	8.42	6.55	10.29
2	15.73	17.74	15.96	17.18	15.96	16.8	15.16	18.59	20.78	13.43	17.83	21.2
4	44.05	36.04	32.25	28.93	32.25	24.67	32.25	35.11	26.54	32.11	30.66	30.71
6	68.82	47.37	44.1	48.64	44.1	33.38	44.1	47.23	38.34	45.83	44.1	39.25

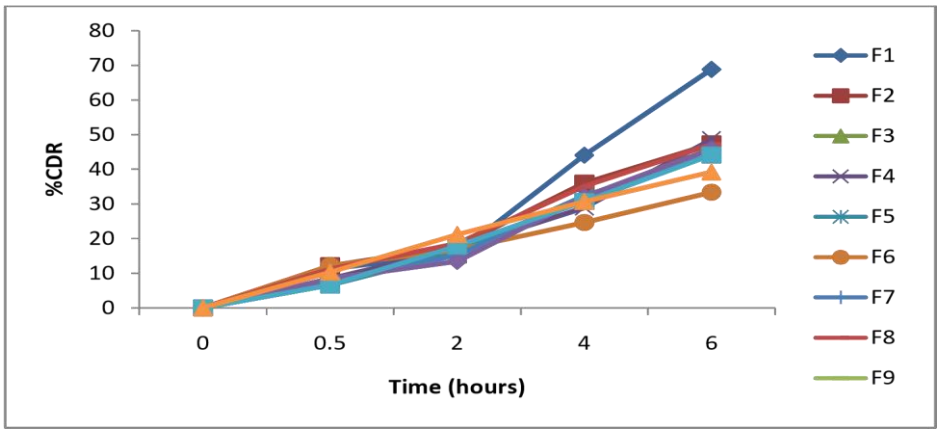

Fig. 8 Comparative Drug diffusion study for all formulations

Evaluation of cubosomal gel

The prepared gel was evaluated for pH, viscosity, Spreadability, Drug content, Washability.

pH

The pH of the prepared gel was determined by digital pH meter and was found to be 7.18 which is optimum for skin's pH range.

Viscosity

Viscosity being an important parameter for transdermal gel as it determines fluid's resistance to flow property and its consistency. Viscosity for optimized cubosomal gel formulation was 320 observed to be 743Cps which is shown in table 5.

Table 5 Determination of Viscosity by Brookefield Viscometer

Sample	Spindle	RPM	Shear rate	Viscosity	FSR%
Gel	02	100	1333	743 ± 0.02	19.8

Mean ± SD (N=3)

Spreadability

Both Spreadability and Viscosity play a vital role in ensuring the patients compliance on topical application of the gel. Spreadability is the ability of the gel to uniformly spread on the skin, which increases patient compliance. The spreadability test is carried out in triplicates and was found to be 11.2±0.28gcmsec^{-1}. All the readings were recorded in triplicate and across various dimensional lengths in table 6.

Table 6 Determination of Spreadability

Sr no.	Line 1	Line 2	Line 3	Line 4	Average	Spreadability gcmsec^{-1}
1	2.7	2.7	2.7	2.9	2.75	11±0.01
2	2.9	2.9	2.7	3.0	2.87	11.48±0.02
3	2.9	2.9	2.7	2.7	2.8	11.2±0.01

Mean ± SD (N=3)

Determination of washability

Washability test for gel was performed and the time taken to completely wash off the gel was recorded as 13 seconds.

Drug Content

The drug content in the gel formulation was found to be 82.40%.

Table 7 Determination of %Drug Content

Sample	Drug Content (%)
F1 gel	82.40

Stability studies

Stability studies such as drug content, pH and drug release rates were analysed for optimized gel formulation. The stability study was performed for 3 months at room temperature. The stability is determined by the values of Drug Content, Drug Release and pH at T_0 (Day1) and T_1 (After 3 months) and the results were recorded in table 8.

Table 8 Estimation of Stability studies for 3 months

Stability studies	Drug content (%)	Drug release (%)	pH
T_0	82.40	70.2	7.18
T_1	82.36	67.3	7.15

Conclusion

Serratiopeptidase loaded cubosomes were developed using excipients GMO as lipid core and Pluronic f-127 as polymer. These cubosomes were evaluated using various characterization techniques and methods. Cubosomes formulation of F1 was selected as optimized formulation containing 0.5% of polymer and 1% of lipid. The optimized formulations characterization was done based on Particle size, Entrapment efficiency, Zeta Potential, PDI and was found to be 200nm, 87.37%, -52.8 and 0.48 respectively. The prepared gel showed an pH suitable for the skin, a good viscosity, spreadability, drug release and drug content. The prepared gel containing cubosomal nanoformulations of Serratiopeptidase that can be viably used to alleviate arthritis, and associated symptoms such as joint inflammation and pain.

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