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Original Article

Quality by Design Assisted Optimization of Gemcitabine Loaded Nanocochleates

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ABSTRACT

Background: Gemcitabine hydrochloride (GEM) is a drug of choice in treatment of various malignancies, but has limited use for oral drug delivery because of its very short plasma half-life. Nanocochleates can be a promising nanocarrier for improving oral delivery of GEM. **Materials and Methods:** The Trapping method was utilised to prepare nanocochleates of GEM. To find a wide range of potential causes influencing particle size and entrapment efficiency, the Ishikawa diagram was employed as a cause analysis tool. The Taguchi screening model was used to filter the variables influencing the particle size. Through central composite design, the important parameters influencing the particle size and entrapment efficiency were chosen for optimization. Optimized GEM loaded nanocochleates were characterized by particle size, zeta potential, X-ray Diffraction (XDR), Differential Scanning Calorimetry (DSC), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM). Optimized nanocochleates were evaluated for GEM release profile and kinetic models. **Results:** The average particle size of GEM loaded nanocochleates dispersion was found to be 39.8 nm and the zeta potential was -24.3 mV. The maximum entrapment efficiency that was achieved was 86.6%. The formulation's tubular structure was verified by transmission electron microscopy and scanning electron microscopy images. The slow release of GEM was demonstrated by the nanocochleates' ability to release the drug over an extended period of time. It was inferred that the release of GEM from nanocochleates followed a non-Fickian diffusion pattern in the Korsmeyer-Peppas kinetic model. **Conclusion:** With better oral delivery and fewer side effects, nanocochleates have shown to be a promising carrier for the anticancer medication GEM.

Keywords: Gemcitabine hydrochloride, Nanocochleates, Optimization, Particle size, Entrapment efficiency, Korsmeyer-peppas kinetic model.

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INTRODUCTION

A fluorinated nucleoside derivative of cytosine arabinoside (Ara-C), Gemcitabine hydrochloride (GEM), is known for its possible anticancer activity.[1] It is primarily indicated as the medicine of choice for treating a variety of malignancies, including pancreatic, non-small cell lung, ovarian, bladder, neck and head cancers.[1] GEM works by preventing DNA synthesis using a chain termination mechanism. GEM is a powerful anticancer drug, but due to its extremely short half-life (short infusions 32 to 94 min, long infusions 245 to 638 min) and highly hydrophilic nature, it is unable to keep an optimal concentration in the body. GEM is quickly converted by the enzyme cytidine deaminase in the body after systemic administration to its inactive metabolite 2',2'difluoro-2'-deoxyuridine.[2]

Main drawbacks of GEM include its short half-life and potential side effects when given intravenously. Fast renal clearance, reduced protein binding and degradation by enzymes are the main causes of its short half-life.[3] It is clearly evident that an alternate route of drug delivery such as the oral route due to its benefits of being non-invasive and patient compliant would be beneficial.[4]

Nanocochleates enhance the oral drug effectiveness of drugs with low oral bioavailability, hence the objective of this research work was to formulate GEM nanocochleates for enhancing the therapeutic efficiency and safety profile of the traditional cancer treatment.[5] Although they haven't been studied as much as liposomes, nanocochleates are newer drug delivery systems with greater benefits. Nanocochleates are lipid-based drug delivery system containing bilayer of lipids rolled to form cylindrical structure.[6,7]

When negatively charged liposomes and cationic salt interact, a sequence of lipid bilayers is created that eventually forms nanocochleates, which resemble cigars. A crucial part of Nanocochleates is phospholipid since it is the primary

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component in the synthesis of nanocochleates. Calcium ions are utilized to fuse phospholipids, resulting in the stacked sheets. Phospholipid's hydrophilic and hydrophobic regions enable it to transport hydrophilic, hydrophobic and amphiphilic medications, indicating the system's multiple applications.[8]

Nanocochleates were prepared by Trapping method[9] and to yield optimized formulation the systematic approach of 'Quality-by-Design' was applied during the formulation studies.[10,11]

MATERIALS AND METHODS

Materials

GEM was received as gift sample from Neon Laboratories Ltd., (Mumbai, India), Phospholipon 90H was received as gift sample from Lipoid (Germany), Leciva S90 was received as gift sample from VAV Life Science (Mumbai), cholesterol was purchased from Loba Chemicals (Mumbai), Chloroform of analytical grade was purchased from Loba Chemicals (Mumbai), Cellulose Dialysis membrane was purchased from Hi Media Lab, (Mumbai).

Methods

Preformulation Study

Preformulation study included physical observation of GEM sample, melting point determination which was done using Thiele tube with capillary. The capillary was sealed from one end was filled with the GEM sample and was immersed in the apparatus with liquid paraffin a thermometer. Temperature range was observed over which the sample melted.

Drug excipient compatibility study

GEM-excipient compatibility was determined for any physiochemical interaction. To asses GEM excipient compatibility pure GEM and excipients were mixed in 1:1 ratio and was kept for 1 month at 25°C±2° and 65% RH±5%. After a month the sample mixtures were evaluated visually and by FTIR.

Analytical method

UV spectrophotometric analytical method development and validation.

Preparation of stock solution

Stock solution of GEM 1000 µg/mL was prepared by dissolving 10 mg of GEM in 10 mL of distilled water and phosphate buffer pH 7.4 respectively in 10 mL volumetric flask. Further dilutions were made from the above stock solution.

Determination of absorbance maxima

From stock solution 1 mL aliquot was withdrawn in 10 mL volumetric flask and diluted to 10 mL with distilled water and phosphate buffer pH 7.4. This resulted into 100 µg/mL solution. From this 1 mL aliquots were taken in 10 mL volumetric flask and diluted with 10 mL of distilled water and phosphate buffer pH 7.4. This resulted into stock solution of GEM 10 µg/mL. UV spectrum of this 10 µg/mL was recorded using UV-Vis spectrophotometer between range of 200-400 nm. λ_{max} of GEM in distilled water and phosphate buffer pH 7.4 was found to be 267 nm and 267.5 nm respectively.

Preparation of calibration plot: From the stock solution of 100 µg/mL aliquots of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 mL were withdrawn and further diluted up to 10 mL with distilled water and phosphate buffer pH 7.4 respectively to obtain concentration range of 2-20 µg/mL. The absorbance of this solution was measured at λ_{max} 267 and 267.5 nm respectively. A concentration vs absorbance graph was plotted.

Method validation

Method validation was done following ICH Q2B guidelines to determine validation parameters of linearity, accuracy, precision, robustness, LOD and LOQ.

Formulation development

Preparation of GEM loaded nanocochleates

Nanocochleates were prepared by trapping method using preformed liposomes.

Method of preparation of liposomes

Phospholipid (Leciva S90 or Phospholipon 90H) and cholesterol in appropriate quantities were dissolved in 5 mL of chloroform to prepare organic phase covered with aluminum foil, GEM solution containing 10 mg of GEM dissolved in distilled 1 mL water was added dropwise to the above mixture whose temperature was maintained at 60°C. The above mixture was stirred with help of magnetic stirrer for 20 min to form primary w/o emulsion. This primary emulsion was then added dropwise to the 15 mL of aqueous phase containing phosphate buffer pH 7.4 to obtain w/o/w emulsion. The resulting mixture was stirred for 30 min to remove the organic solvent. Subsequently the dispersion was then sonicated for 15 min.

Nanocochleates were formed by trapping method. Previously prepared liposomes were converted into nanocochleates by addition of 0.1M calcium chloride solution under vortex.

Optimization by DOE

A response surface design using Design-expert® software version 12 was employed to optimize critical formulation and process factors.

Ishikawa diagram as shown in Figure 1 was used to identify the causes of the events.

To better understand the variables of nanocochleates formulation principles of QbD were applied. The following factors were identified:

Quality Target Product Profile (QTTP) for GEM loaded nanocochleates

As per Q8 ICH guidelines following QTPP parameters of GEM loaded nanocochleates were deemed significant as dosage form, dosage design and route of administration.

Critical Quality Attributes (CQA)

Quality attributes of GEM loaded nanocochleates were physical attributes naming the characteristics of appearance, particle size and percent entrapment efficiency that were deemed to be important for the quality of the GEM loaded nanocochleates as shown in Table 1.

Risk assessment

Risk assessment of nanocochleates attribute was done to analyze the effect that each attribute could have on CQAs of GEM loaded nanocochleates.

Each attribute was assigned a high, medium, or low relative risk rating. Attributes affecting nanocochleates formulation are enlisted as type of lipid, GEM lipid ratio, aqueous to organic phase ratio, lipid cholesterol ratio, volume of calcium chloride and stirring speed.

Formulation of Taguchi screening batches of GEM loaded nanocochleates

Batches of GEM loaded nanocochleates were formulated using 2^7 screening model. Factors X1(type of lipid), X2 (GEM lipid ratio), X3(lipid cholesterol ratio), X4 (volume of calcium chloride), X5 (aqueous phase to organic phase ratio), X6 (stirring speed), X7(probe sonication time).

Formulation of central composite design batches of GEM loaded Nanocochleates (NCS)

Most significant factors which were showing major effect on particle size and entrapment efficiency of GEM loaded nanocochleates were identified using Taguchi screening design. The screened factors S1 (GEM lipid ratio) and S2 (probe sonication time) were optimized using 2^2 central composite design.

Freeze-drying

To prevent GEM loaded nanocochleates from aggregation, leakage and fusion of encapsulated GEM in dispersed medium nanocochleates were freeze dried using lab tech freeze dryer at 40°C for 24 hr. temperature and vacuum conditions were optimized to facilitate complete recovery of dried nanocochleates.

Characterization

The GEM loaded nanocochleates were characterized for following parameters

Particle size

Particle size and polydispersity index of GEM loaded nanocochleates were measured using Dynamic Light Scattering (DLS)method using Horiba particle size analyzer. The measurement of variations in scattered light intensity over time is known as dynamic light scattering. A 4.5-mW laser diode operating at 670 nm was used as the light source for the studies and size measurement was done at a scattering a 90° angle. Double distilled water was used to dilute the samples. The Measurements were made in triplicate at 25°C.[12]

Zeta potential

Dispersion of nanocochleates was diluted with double distilled water and zeta potential was determined using Horiba zeta potential analyzer. It transforms optical signals into zeta potential information using signal processing circuits. The average zeta potential and charge of GEM loaded nanocochleates were found and the analysis period was limited to one minute. Three runs were conducted at a temperature of 25°C.[13]

X-ray diffraction (XRD)

XRD study was used to obtain information regarding crystalline structure, physical properties and chemical composition of material. Pure GEM and Lyophilized product of optimized batches of GEM loaded nanocochleates were irradiated with X-ray radiations using XRD and analyzed between 10° to 80°.[14]

Differential Scanning Colorimetric (DSC)

Thermograms of pure GEM and GEM loaded nanocochleates were obtained using DSC. hermetically sealed powder sample in aluminum pan sample holder were analyzed at constant rate 10°C, over a temperature range of 35°C-300°C. nitrogen purging at a flow rate of 150 mL/min was used to maintain the atmosphere.[15]

Fourier Transformed Infrared Spectrophotometry (FTIR)

Chemical composition of GEM loaded nanocochleates was evaluated using FTIR. Sample was placed on sample holder and was analyzed for functional groups by scanning at 200 to 4000 cm^{-1}. The functional groups at reported wavelength confirmed about the GEM loading in nanocochleates.[15]

Field Emission Gun-Scanning Electron Microscopy (FEG-SEM)

Surface morphology and shape of GEM loaded nanocochleates were observed using scanning electron microscopy. Sample was coated with gold coating and then subjected to reduced pressure with ion spluttering device. The gold coated nanocochleates were observed under SEM and images were taken.[16]

Transmission Electron Microscopy (TEM)

Size, surface morphology and shape of GEM loaded nanocochleates were observed using Transmission electron microscopy. One drop of GEM loaded nanocochleates dispersion was applied to a copper grid to prepare the sample, which was afterwards dried at room temperature before being dyed with phosphate tungsten acid. TEM was then used to analyze the sample at 120 kv. Image analysis was done by using Soft Imaging and Digital Micrograph software.[17]

Determination of GEM entrapment efficiency (%) and GEM loading (%)

GEM loaded nanocochleates were taken in Eppendorf tubes and centrifuges using cold centrifuge at 15000 rpm at 2-4°C for 45 min. nanocochleates get settled down at the bottom of Eppendorf forming a pellet. Supernatant was separated and amount of GEM in the supernatant was estimated by taking absorbance at 267 nm and 267.5 nm respectively for GEM using UV spectrophotometer.[18]

% Entrapment efficiency was calculated using following formula:

$$\%EE = \frac{(Total_{gem} - Unent_{gem})}{Total_{gem}} \times 100$$

In vitro GEM loaded nanocochleates release study

5 mL of GEM loaded nanocochleates dispersion and GEM solution was filled in activated cellulose dialysis bag with both the ends sealed with closure clips. The dialysis bag was immersed in 100 mL of phosphate buffer pH 7.4. GEM release medium was maintained at 37±0.5°C and was stirred continually using magnetic stirrer kept at 100 rpm. 1 mL of aliquot was withdrawn at predetermined time intervals of 0, 1, 2, 3, 4, 5, 6, 7, 8 and 24 hr. Equal volume was replenished with fresh medium after each sample withdrawal. The concentration of GEM released over a period of time was estimated using UV spectrophotometer.[19]

Table 1: **Critical quality attributes for GEM loaded nanocochleates.**

Quality attribute of GEM nanocochleate		Target	CQA (Yes/No)	Rationale
Physical attributes	Appearance	Accepted for color and odor	No	Since colour, odour and appearance are not closely related to efficacy and safety, they are not regarded as crucial.
	Particle Size	Size of nanocochleates would need to be in nano.	Yes	Nanocochleates' sustained release and cell entry are caused by their small size.
Percent entrapment efficiency		60-95%	Yes	Percent entrapment efficiency is crucial while adjusting the dose of formulation.

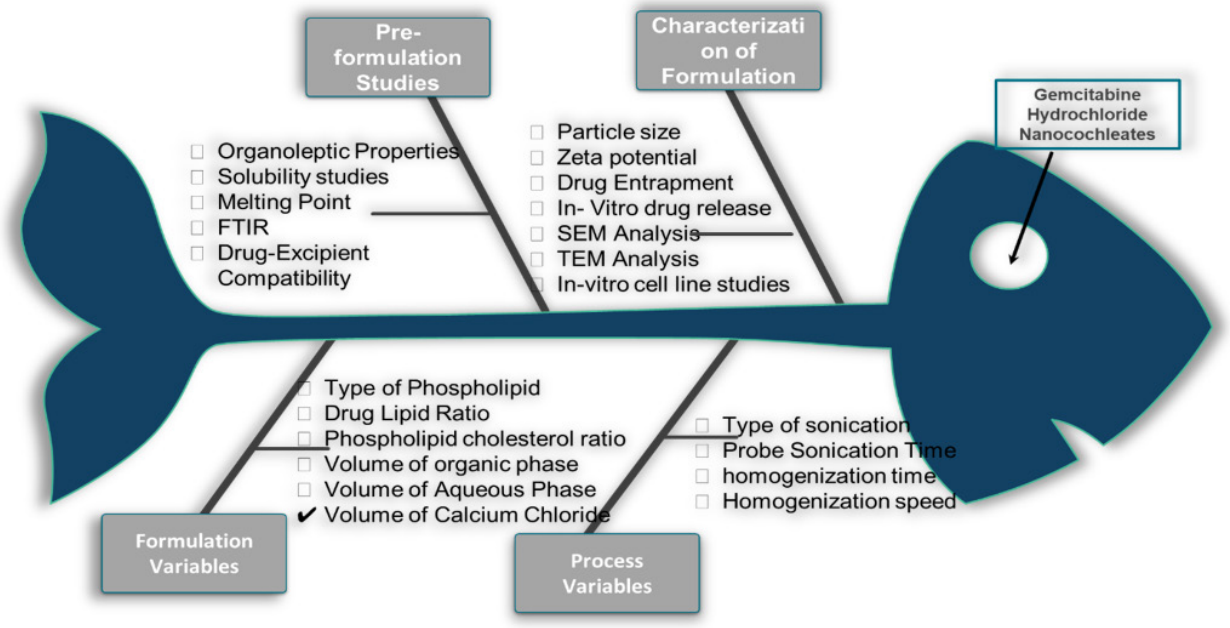

Figure 1: Ishikawa diagram for cause analysis of GEM loaded Nanocochleates.

RESULTS

Determination of absorbance maxima of GEM in distilled water and in Phosphate Buffer (pH 7.4).

The absorbance maxima of GEM were found to be at 267.5 nm and 267 nm respectively in a) Distilled water, b) Phosphate buffer (pH 7.4).

Compatibility study

Drug recovery and physical observation yielded satisfactory results from the drug compatibility study with excipients. Compatibility study showed no significant changes in physical appearance of pure GEM and GEM excipient mixture as shown in Figure 2(a) and (b) respectively. GEM showed characteristic peaks at 3387, 2939, 1675 and 1053 cm^{-1} which represent N-H group and CO-NH group. FTIR spectra of physical mixture of GEM with excipients showed the characteristic peaks of pure GEM indicating no change in chemical nature of GEM.

Percent Entrapment efficiency and particle size

Entrapment efficiency and particle size of batches prepared for screening model is as per Table 2. Response is evaluated for different parameters such as half normal plot, Pareto chart, ANOVA, counter plot and 3D surface plot.

Optimization of GEM loaded nanocochleates by central composite design

Prepared batches of optimization model were evaluated for % entrapment efficiency and particle size as shown in Table 3.

Percent entrapment efficiency of formulation batches was found to be in 50%-87% range. F6 batch was found to have highest %entrapment efficiency of 86.6%. average particle size of GEM loaded nanocochleates were found to in rage of 30 nm-350 nm. F6 batch was found to have minimum particle size of 39.8 nm.

The formulation was optimized for dependent variable S1 (% Entrapment Efficiency) and S2 (particle size). The F6 batch

Table 2: % Entrapment efficiency and particle size of Taguchi screening design.

Batch code	% Entrapment efficiency	particle size (nm)
NCS 1	80	570.2
NCS 2	78	1949
NCS 3	55	784.5
NCS 4	84	529.3
NCS 5	59	2809
NCS 6	79	1101
NCS 7	60	517.8
NCS 8	61	2212

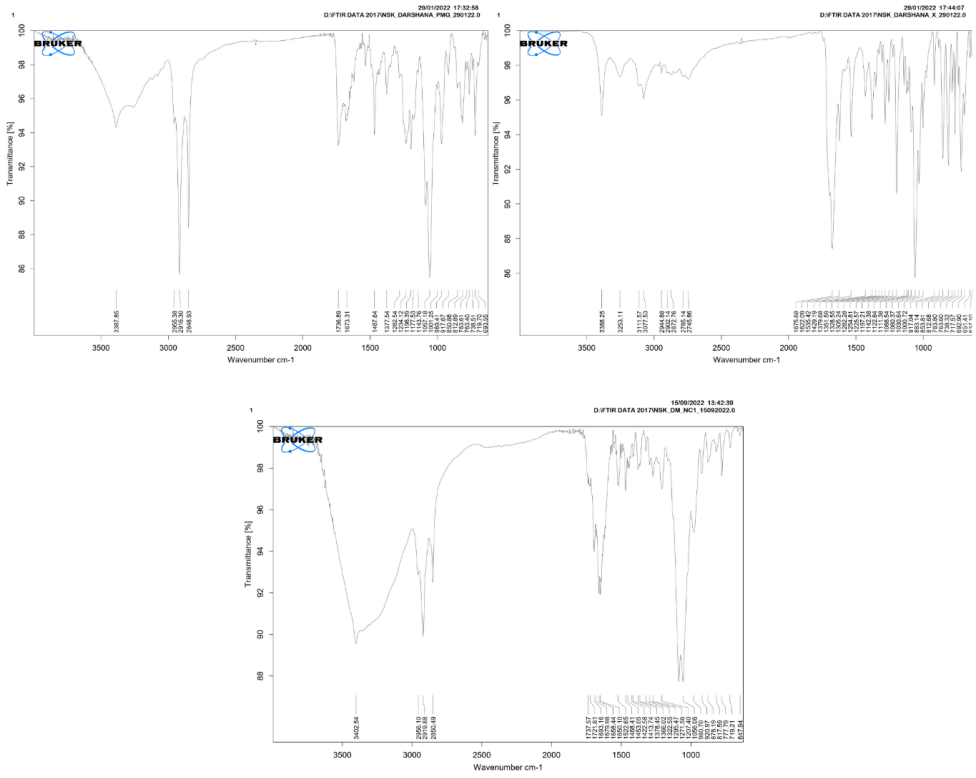

Figure 2: FTIR Spectra (a) Pure GEM, (b) Physical mixture of GEM with excipients, and (c) GEM loaded Nanocohleates optimized batch.

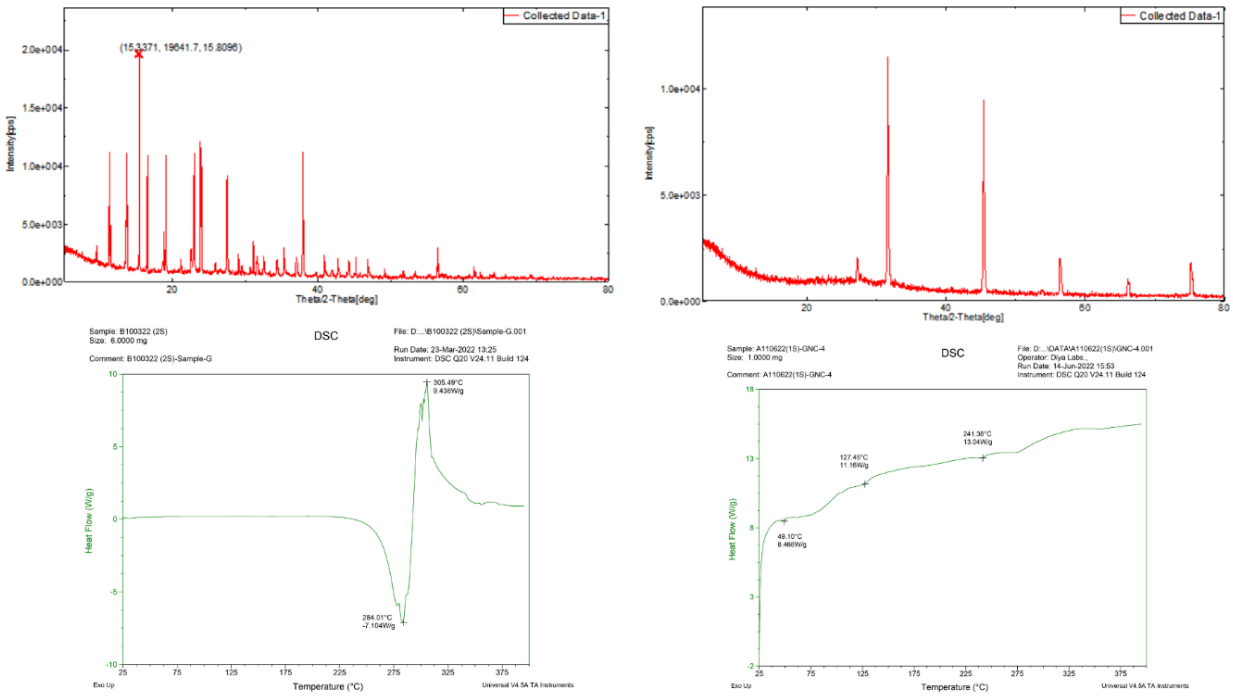

Figure 3: XRD graph of (a) pure GEM, (b) GEM loaded Nanocochleates optimized batch and DSC graph of (c) pure GEM, (d) GEM loaded Nanocochleate optimized batch.

obtained out of 13 batches which was having minimum particle size and maximum entrapment efficiency. The results of optimized formula were compared with the predicted values.

Fourier Transformed Infrared Spectrophotometry (FTIR) of optimized batch of GEM loaded nanocochleates

FTIR of optimized batch as per Figure 2(c)was showing all the characteristic peaks of GEM observed in FTIR spectra of GEM as shown in Figure 2(a) which states the GEM is efficiently being entrapped in nanocochleates.

Particle size and polydispersity index

particle size of GEM loaded nanocochleates with polydispersity index before freeze drying was found to be 39.8 nm and 0.28 respectively. Whereas particle size of GEM loaded nanocochleates with polydispersity index after freeze drying was found to be 128.7 nm and 0.27 respectively.

Zeta potential

Zeta potential of optimized batch was found to -24.3 mv. which was in the acceptable range of +/-30 mv. Hence it states that the optimized batch was found to be stable.

Percent Entrapment efficiency

Percent entrapment efficiency of optimized batch of GEM loaded nanocochleates was found to be 86.6%.

Table 3: Observed response for % Entrapment efficiency and particle size of central composite optimization design.

Batch code	% Entrapment efficiency	particle size (nm)
F1	54.2	66
F2	74	45.3
F3	71	65.7
F4	55	59.4
F5	63	284.5
F6	86.6	39.8
F7	52.5	221.3
F8	79	95.5
F9	84.7	328.6
F10	80.7	44.9
F11	60	44.2
F12	53.5	48.9
F13	73.3	303.9

X-ray Diffraction (XRD)

XRD graph of pure GEM as shown in Figure 3(a) when compared with the XRD graph of pure GEM loaded nanocochleates as shown in Figure 3(b) it showed that there was significant reduction in sharp peaks as compared to pure drug which confirmed the amorphous nature.

Differential Scanning Calorimetry (DSC)

DSC of pure GEM as shown in Figure 3(c) when compared with the DSC graph of GEM loaded nanocochleates as shown in Figure 3(d) showed that there was no endothermic peak as compared to pure GEM which confirmed that the GEM is successfully encapsulated in nanocochleates.

Field Emission Gun-Scanning Electron Microscopy (FEG-SEM)

FEG-SEM image of GEM loaded nanocochleates as per Figure 4(a) was found to show cylindrical cigar like structures of nanocochleates which confirmed the formation of nanocochleates.

Transmission Electron Microscopy (TEM)

Particle size, surface morphology and shape of nanocochleates were analyzed using TEM. It was observed as per Figure 4(b) the nanocochleates were cylindrical in shape and they were well dispersed. Particle size was found to be below 50 nm. The obtained particle size could aid the nanocochleates for improved oral delivery of GEM.

Cumulative Drug release study

Drug release study of pure GEM solution was compared with the drug release of GEM loaded nanocochleates. It was seen that the pure GEM solution showed 90% drug release at the end of 8 hr whereas nanocochleates showed 70% drug release at the end of 8 hr which confirmed the sustained release of GEM over long period of time. Graph of % Cumulative release versus time was plotted.

Kinetics Study

Cumulative drug release data was studied for kinetic model fit by zero order, first order, higuchi model and korsemeyer peppas model and it was found that the Korsemeyer peppas model showed a regression coefficient near to 1 which stated that the optimized batch of GEM loaded nanocochleates showed non-fickian diffusion model and followed korsemeyer peppas model kinetics. zero order kinetic model, First order kinetic model, Korsemeyer-peppas model, Higuchi model were studied.

The comparative data for regression coefficients of various kinetic models was found to be as shown in Table 4 and it was confirmed that the nanocochleates drug release followed Korsemeyer-peppas kinetics as it had a regression coefficient closest to 1.

DISCUSSION

The research work was initiated with preformulation studies of GEM and the excipients. Stable formulation development of GEM warranted the screening of formulation and process factors that affected the entrapment efficiency and particle size of nanocochleates using the Taguchi screening model. Nanocochleates were formulated using the trapping method. Initially, small unilaminar vesicles, i.e., liposomes, were formed and then they were converted into nanocochleates by the trapping method using calcium chloride.

After evaluating the Taguchi screening model for selected factors, it was found that two factors, drug lipid ratio and probe sonication time, were most significantly affecting entrapment efficiency and particle size; hence these two factors were selected for optimization of formulation. Central composite design was

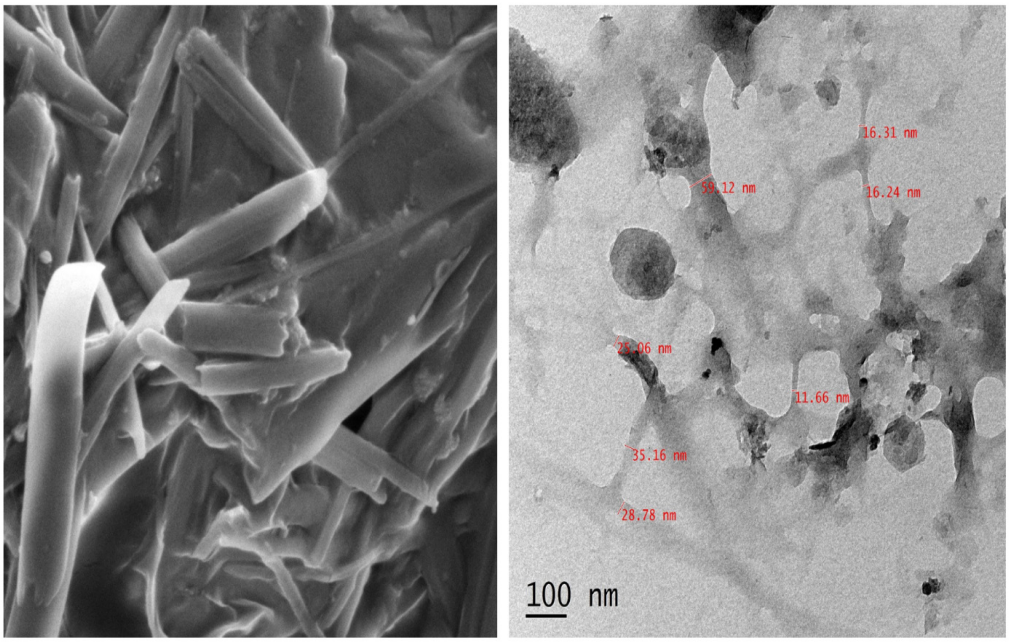

Figure 4: FEG-SEM image of (a) GEM loaded Nanocochleate optimized batch and (b)TEM image of GEM loaded Nanocochleate optimized batch.

Table 4: Optimized batch of GEM loaded nanocochleates release kinetic data.

Model	Zero order	First order	Higuchi	Korsemeyer-peppas
R^2	0.9494	0.7705	0.9726	0.9997

used for optimization of screened factors. Based on the highest entrapment efficiency and smallest particle size Batch F6 was selected as the optimized batch. The particle size of the optimized F6 batch was found to be 39.8 nm and entrapment efficiency was found to be 86.6%. Blood vessel hole sizes in tumour tissues are between 400 and 600 nm in size. Because of this, in order for the carrier system to reach the tumour tissue, its particle size needs to be 200 nm or smaller.[20]

The zeta potential of the optimized batch F6 was found to be -24.3 mV, which indicates good stability. To prevent agglomeration, the nanoparticles need to possess a specific zeta potential, which is documented as ±30 mV.[21] These outcomes demonstrated an effective formulation design.

Optimized batch F6 was further characterized by FEG-SEM and TEM to confirm the morphological structure of nanocochleates. A cylindrical, cigar-like shape was observed with a particle size of less than 50 nm. Entrapment of GEM in nanocochleates was further confirmed by XRD and DSC studies.

The cumulative drug release study showed that 70% of GEM was released from nanocochleates at the end of 24 hr. when compared with the drug release study of GEM solution, which showed 90% drug release at the end of 8 hr, confirming the sustained release of GEM. *In vitro* release profiles offered insights into the behaviour and structure of the formulation, potential interactions between the drug and the carrier system and their impact on the rate and mechanism of GEM release.[22] Cumulative drug release data of optimized batch F6 of GEM-loaded nanocochleates was studied for different kinetic models. R^2 values of zero order, first order, Higuchi and Korsmeyer-Peppa models were found to be 0.9494, 0.7705, 0.9726 and 0.9997, respectively. The optimized GEM-loaded nanocochleates demonstrated non-Fickian diffusion release and it was confirmed that the optimized GEM-loaded nanocochleates followed the Korsmeyer-Peppas kinetics as it had a regression coefficient close to 1. Hence the present work confirmed the development of a stable sustained release GEM nanocochleates for its oral delivery.

CONCLUSION

The research work successfully developed stable and sustained release nanocochleates for the oral delivery of GEM with the strategy of QbD assisted optimization of the GEM nanocochleates formulation. It can be concluded that the nanocochleates were a promising delivery system with notable enhancement of GEM entrapment and achievement of sustained release of GEM over for a period of 24 hr.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

GEM: Gemcitabine hydrochloride; **FEG-SEM:** Field Emission Gun Scanning Electron Microscope; **TEM:** Transmission Electron Microscope; **DSC:** Differential Scanning Colorimetry; **XRD:** X-ray Diffraction; **FTIR:** Fourier Transformed Infrared Spectroscopy.

SUMMARY

The objective of this research was to develop a nanosized lipid drug delivery system with enhanced bioavailability and improved stability for the oral administration of GEM, a medication categorized as BCS class III but having very low oral bioavailability and short half-life. The GEM loaded nanocochleates were created by using the Trapping technique, which was then refined using Central Composite Design (CCD) to produce the ideal formulation properties.

Comparing the drug release profiles of the optimized GEM loaded nanocochleates formulation to those of the pure GEM, it was found the nanocochleates gave a sustained drug release noticeably better than only GEM. The sustained release was attributed to enhanced absorption due to the lipid-based nanostructure's characteristics. All things considered, the study demonstrated the effectiveness of naocochleates for oral GEM delivery, providing a viable solution to the problems of plasma degradation and bioavailability. Hence nanocochleates were found to be a promising nanosystem for improved bioavailability.

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