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Research Article

Development of Self-Nano-Emulsifying Drug Delivery System for Gemcitabine: *In Vitro* and *Ex Vivo* Evaluation

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Abstract

Researchers often aim to develop novel drug delivery systems to overcome the barriers associated with conventional drug delivery systems. Therefore, GEM (Gemcitabine), an anti-cancerous drug, belonging to the Biopharmaceutics Classification System (BCS) Class III with reduced permeability, is an important challenge. Self-nano-emulsifying drug delivery system (SNEDDS) was opted to be designed in such a way that it improves the permeability of GEM. From compatibility and solubility studies, Kolliphor RH40, castor oil, and polyethylene glycol-400 were chosen in optimized ratios for preparation into GEM-SNEDDS. GEM-SNEDDS F3 showed -12.1±6.83 mV zeta-potential, 0.406 PDI, and 94.81±82.73 nm particle size when tested for *in vitro* and *ex vivo* studies, F3 showed improved GEM release in simulated gastric fluid and simulated intestinal fluid *in vitro* and enhanced permeability *ex vivo*. These factors are prerequisites for the improvement of poor bioavailability of GEM. From stability studies, it was evident that F3 retained its physical stability at several stability conditions and in drug encapsulation and loading. Hence, we developed GEM-SNEDDS as a novel drug delivery system for increasing the permeability and, in turn, the oral bioavailability of GEM.

Keywords: Gemcitabine, self-emulsification, solubility, self-emulsifying drug delivery system, nano-emulsion

1. Introduction

Oral Drug delivery systems are a significant challenge in healthcare, especially in cancer treatment due to several factors including the intrinsic properties of the drug, its solubility, etc. Nanotechnology has become a focal player in tackling this problem and has helped researchers overcome challenges of poor solubility and permeability. Self-nano-emulsifying drug delivery systems (SNEDDS) which are homogenous lipid-based systems or concoctions containing drug solubilized in surfactants and oil phase are relatively new drug delivery systems. SNEDD is considered to be a futuristic delivery system for multiple drugs due to its simplicity, manufacturing ease, and delivery. Various methodologies have been adopted by researchers to formulate and optimize SNEDDS and improve the oral delivery of anti-cancer drugs. Across the globe, researchers and/or scientists have paid greater attention is designing multiple approaches to enhance the drug's bioavailability using SEDDS (Xue et al. 2018), for the successful delivery of anti-cancerous drugs through the oral route. The majority of the data supports the claim that SNEDDS significantly boost permeability, solubility, and, therefore, the bioavailability of hydrophobic or lipophilic anti-cancerous drugs (Shukla et al. 2023).

A prevalent malignancy in Western countries, breast cancer is estimated to have caused 685,000 deaths globally in 2020 (2023). Combined chemotherapeutic agents and hormonal adjuvants improve overall and disease-free survival for

patients with early stages of illness. However, many patients experiencing this disease need systemic medications (chemotherapy, modern biological agents, hormone therapy, and other treatment options) for symptomatic relief and an increase in life span or quality of life enhancement. Ultimately, the therapeutic index of a particular drug/chemotherapeutic agent or treatment plan needs to be carefully thought through. In this context, the recognition of chemotherapeutic agents/drugs that are both highly effective and well-tolerated is of great interest.

Cancer therapy has been a significant challenge for pharmaceutical research, delivery experts, and practitioners. Chemotherapeutic agents may also affect non-cancer cells, which sometimes results in unmanageable toxicity and worsening of the disease, and is a fundamental disadvantage of utilizing them to treat cancer. As a result, a large number of patients pass away from unmanageable metastatic disease or from failing to respond to conventional chemotherapeutic treatments. Therefore, the goal of the safe and efficient treatment of tumors is to direct the drug to targeted tissues while avoiding toxicity by reducing its reach to normal and healthy cells. By modifying drugs or creating targeted and/or site-specific delivery systems, the field of drug delivery and development has recently achieved major milestones. These systems have been shown to significantly enhance cancer treatment (Reddy and Couvreur 2008).

Gemcitabine (GEM) has a shorter plasma $t_{1/2}$ half-life (2-6 hours) and low absorption, which necessitates repeated treatment, leading to severe adverse effects such as myelosuppression, nephrotoxicity and/or hepatotoxicity but still fails to achieve required therapeutic effect. Consequently, it is necessary to develop an effective drug delivery system utilizing strategies that promote its solubility and permeability to overcome this issue without increasing the frequency and/or severity of adverse effects.

The present study was aimed at formulating and characterizing oral GEM-loaded SNEDDS to improve oral bioavailability and intestinal permeability. Based on *in vitro* and *ex vivo* studies, we were able to provide an alternative way for resolving issues regarding the half-life of GEM, dosing, and toxicities related to it.

2. Materials and Methods

2.1. Materials

Gemcitabine (GEM) was purchased from Sigma Aldrich, St. Louis, Mo, USA. Tween 80 (polyoxyethylene sorbitan monooleate) was purchased from Hangzhou Zhongbao Imp. & Exp. Corp. Ltd, Hangzhou, China, Tween 20 from Avantor ™ Performance Materials Inc, PA, USA, Span 20, 80 (sorbitan monooleate) from Techno Pharmchem, Phase III, Delhi, India, Corn oil from Acros organics, Janssen Pharmaceuticalaan 3A, Geel, Belgium, Sodium Lauryl Sulphate (SLS) from Shanghai Auway Daily Chemicals Co., Ltd, China, Cinnamon oil (oil of *Cinnamomum zeylanicum*) and Clove oil from Brighton, BNI 3TN, UK, Propylene glycol (1,2-propanediol) was purchased form Shinghwa Amperex Technology (Dongying) Co. Ltd. China, Polyethylene glycol (PEG)-400 (polyethylene glycol) from Hangzhou Lingeba Technology Co. Ltd., China. Castor oil was purchased from Astral Ltd., India, Kolliphor RH40 (polyoxyl 40 hydrogenated castor oil), Kolliphor EL, and olive oil from Agricultural & Environmental Testing & Research Laboratories, South Africa. No further purification of chemicals was done prior to use, as all chemicals were of analytical grades.

2.2. Pre-Formulation Development

2.2.1. Calibration Curve of Gemcitabine (GEM)

The preparation of the drug solution as standard stock was carried out in a volumetric flask. 1mg of GEM was dissolved in 1mL of ethanol in an Eppendorf tube to obtain a concentration of 1000 µg/mL. Sonication was carried out for

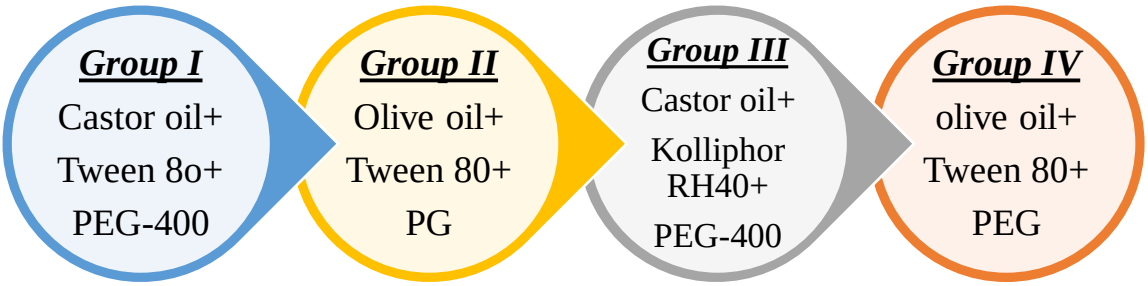

Figure 1. Groups of oil, Surfactants and Cosurfactant for Emulsification Test producing for different combinations of oil+Smix.

Table 1. Different surfactant-cosurfactant mixtures (Smix) with weight ratios.

Surfactant-Cosurfactant Mixtures (Smix)	Smix Code with Weight Ratios
Tween 80:PEG	T-PEG (1:1, 2:1, 3:1)
Tween 80:PG	T-PG (1:1, 2:1, 3:1)
Kolliphor RH40:PEG	K-PEG (1:1, 2:1, 3:1)

approximately 5 minutes for complete solubility of the drug. From the freshly prepared stock solution, further serial dilutions were made with ethanol to get working standard solutions of 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL and 1.5625 µg/mL concentrations. The assay was conducted using UV spectrophotometer at 268 nm as λ_{max} at room temperature. The solutions were immediately analyzed to avoid any degradation. The readings were plotted and the value for R^2 was obtained. Triplicate measurements were taken to minimize errors.

2.2.2. Screening of Excipients Based on Compatibility Studies

Compatibility between excipients, surfactants, co-surfactants, and oils was determined by the method used previously (Jianxian et al. 2020). Mixtures of oil, surfactants, and co-surfactants at selected weight ratios were made in glass vials and stirred thoroughly on a magnetic stirrer at ambient temperature. Visual examination was done and appearances of resulting mixtures were noted.

2.2.3. Selection of Excipients Based on Solubility Studies of GEM

All the excipients of the optimized SNEDDS formulations were selected based on the maximum solubility of GEM in compatible excipients found in the previous step. To find out the appropriate compositions of SNEDDS, the solubility of the drug in multiple excipients was measured. An excess amount of GEM or an increasing amount of GEM was added into 5 mL of each vehicle in separate glass vials. The mixtures were then blended in a shaking water bath at 37°C, 100 rpm for 72 hours for thorough mixing and homogenization, followed by sonication for 20 min and centrifugation at 3000 rpm for 10 min. After achieving equilibrium, a PTFE membrane filter (pore size 0.45 µm) was used to filter the supernatants. The amount of

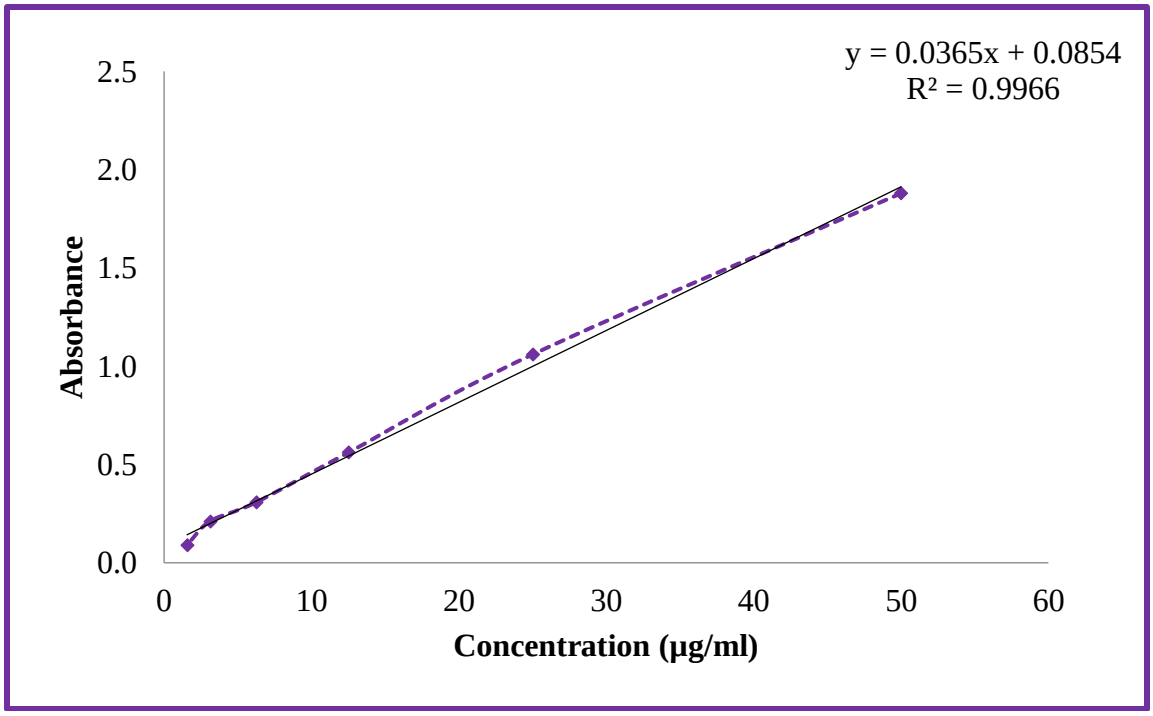

Figure 2. Calibration curve of Gemcitabine in ethanol at 268 nm. R^2 = 0.9966.

GEM loaded was then quantified by UV-spectrophotometry at 268 nm with suitable dilutions by ethanol (Mahmood et al. 2023).

From all the excipients, olive oil and castor oil (oils) were chosen to be the oil phase, Tween 80 and Kolliphor RH40 to be surfactants, and PEG-400 and propylene glycol (PG) to be co-surfactants. Four combinations of selected lipid excipients were analyzed to select the optimal mixture for drug delivery. Figure 1 shows four group combinations that were further experimented.

The selected surfactants-cosurfactants were blended at ambient temperature in weight ratios of 1:1, 2:1, and 3:1 (with surfactant concentration increasing), making surfactant-cosurfactant mixtures (Smix), also known as Km, to determine the effect of Smix ratios on nano-emulsion formation (Jianxian et al. 2020) as described in Table 1. Oil and Smix were thoroughly stirred at different weight ratios (1:1 till 1:9) in separate glass vials preparing 45 new mixtures (Supplementary data).

2.2.4. Assortment of Excipients Based on Self-Nano Emulsification Efficiency

The mixtures (2 mL) (oil+Smix) were slowly stirred, using a magnetic stirrer, avoiding any bubble formation while the addition of the aqueous phase was done using a micropipette. To arrange multiple surfactants and co-surfactants based on solubility studies, briefly, 10µL of liquid mixtures/systems were added dropwise into 100 mL of distilled water in separate beakers (37˚C±0.5˚C, 100 rpm) (Supplementary data). Any formed bubbles were removed by sonication for 1-2 minutes for better visual assessment. The resultant emulsions were visually assessed for the rate of emulsification and appearance and were graded as nano-emulsions based on the following criteria of the grading system:

1. Nano-emulsion (oil in water) (NE): clear, transparent, isotropic emulsion, easily flowable.
2. Nano-emulsion gel (NEG): clear and highly viscous gel-like emulsion.

Table 2. Solubility of Gemcitabine in different excipients

Excipient Categories	Excipients	Solubility (mg/mL±SD)
Oils	Castor Oil	0.712±0.61
	Olive Oil	0.628±0.45
	Corn Oil	0.1038±0.28
	Cinammon Oil	0.023±0.33
	Clove Oil	0.079±0.68
Surfactants	Tween 80	0.4583±0.397
	Kolliphor RH-40	0.2937±1.050
	Kolliphor RL	0.01138±0.918
	Sodium lauryl sulphate	0.049±1.1
	Span 20	0.053±0.99
	Span 80	0.069±1.4
Co-surfactants	PEG-400	0.493±0.996
	PG	0.298±0.238

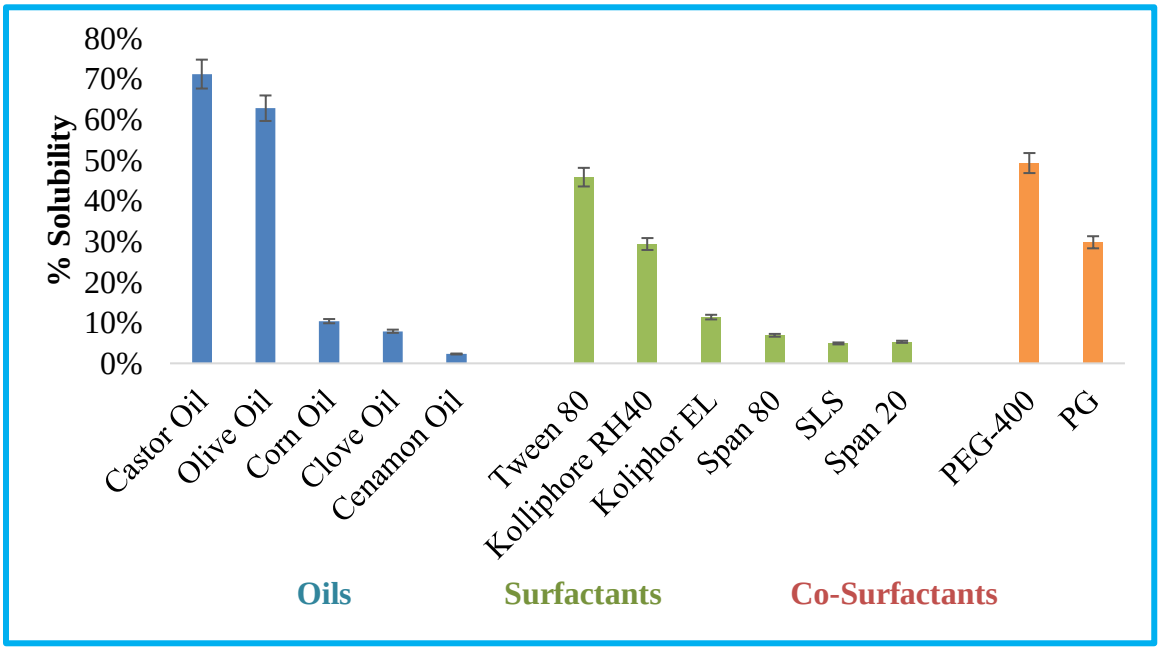

Figure 3. Comparative solubilities of GEM in different components (oils, surfactants and co-surfactants) evaluated at 25°C.

3. Microemulsion (ME): milky, cloudy mixture or nearly transparent homogenous emulsion without phase separation.
4. Emulgel (EG): milky and highly viscous gel (Ansari et al. 2023, Kim et al. 2023, Jianxian et al. 2020).

Systems that showed nano-emulsion upon dilution were then assigned for determination of self-emulsification time. The self-emulsification efficiency was tested using the same protocols as for the determination of nano-emulsions (section 2.2.3). 50µL of selected systems from the previous step were stirred in 100 mL distilled water (37±0.5°C, 100 rpm) on heating magnetic stirrers. Visual examination of the systems was carried out in order to determine the time taken for the

Table 3. Grading of systems for different ratios of all four group combinations at ambient temperature.

Sr.#	Group I		Group II		Group III		Group IV	
	Time (sec)	Visual Grade	Time (sec)	Visual Grade	Time (sec)	Visual Grade	Time (sec)	Visual Grade
1	50	Clear	5	Turbid	1 min	Clear	>1min	Clear
2	35	Clear	17	Turbid	58	Clear	55	Clear
3	49	Clear	15	Turbid	20	Turbid	59	Clear
4	22	Clear	14	Turbid	23	Turbid	25	Turbid
5	45	Clear	6	Gel	25	Turbid	27	Turbid
6	20	Turbid	12	Gel	13	Turbid	29	Turbid
7	**14**	**Clear**	**5**	**Clear**	**11**	**Clear**	**15**	**Clear**
8	16	Turbid	8	Clear	28	Gel	13	Turbid
9	10	Gel	6	Gel	14	Gel	09	Gel
10	8	Gel	o9	Gel	16	Gel	08	Gel
11	11	Gel	11	Gel	7	Gel	17	Gel
12	28	Gel	05	Gel	10	Gel	08	Gel

formation of a homogenous mixture or complete disappearance of pre-concentrates and final remarks for systems were made on the following grading:

- Grade A: clear nano-emulsion (<1 min) with rapid emulsification
- Grade B: translucent/less clear nano-emulsion (>1 min) with rapid emulsification
- Grade C: clear or slightly translucent microemulsion with bluish-white appearance (within 2 min or <1min) with rapid emulsification
- Grade D: bright milky/white emulsion (longer than 2 mins) with slow emulsification
- Grade E: emulsions with either poor emulsification possessing large oil globules on the surface or no emulsion formation (Nasr, Gardouh, and Ghorab 2016, Ansari et al. 2023, Jianxian et al. 2020).

2.3. Development of GEM-loaded SNEDDS

Based on the pre-evaluated combination systems, liquid GEM-SNEDDS were prepared. Each 1 mL of the accurately weighed system was loaded with 1 mg of GEM, and the concentration of the drug was kept constant (0.5 mg/mL). The systems were homogenized thoroughly in glass vials to dissolve the drug completely by continuous stirring at 1200 rpm at 37±0.5°C for 15 mins (Table 3). Filtration was carried out for GEM-SNEDDS using a PTFE 0.45µm membrane filter prior to further analysis to ensure complete removal of un-entrapped drug and stored at 25°C for further use.

2.4. Physicochemical Characterization

2.4.1. Encapsulation Efficiency and Drug Content Analysis

In order to determine the encapsulation efficiency and drug content, GEM was extracted from SNEDDS by diluting one part of GEM-SNEDDS with nine parts of diluent ethanol or as necessary to obtain the required concentrations. Prior to analysis, filtration from PTFE 0.45 membrane filters was done. Analysis was made on a UV spectrophotometer at λ_{max} 268 nm by using ethanol as blank. Each sample was analyzed in triplicate. These GEM-SNEDDS were then kept at ambient temperature for 48 hrs for observation of phase separation or turbidity (cloudiness) before further characterization studies (Ansari et al. 2023, Kim et al. 2023).

$$\%\ Entrapment\ efficiency = \frac{total\ drug - free\ drug}{total\ amount\ of\ drug\ added} \times 100$$

$$\%\ Drug\ loading = \frac{total\ amount\ of\ drug\ entrapped}{drug\ loaded\ nanoemulsion\ weight} \times 100$$

2.4.2. Analysis of Droplet Size, Zeta-Potential, and Polydispersity Index (PDI)

The droplet size, PDI, and zeta potential analysis for optimized GEM-SNEDDS was performed by Zetasizer ZS 90 (Malvern Instruments, Malvern, Worcestershire, UK). Nano-emulsions were prepared by diluting 100µL of GEM-SNEDDS formulation and were made up to 1mL (1:100) with distilled water. The detection range was from 2 to 2000 nm. Each sample was analyzed in duplicates.

2.5. *In Vitro* Release Behavior of Loaded GEM-SNEDDS

GEM-SNEDDS formulations were evaluated for drug release using a shaking water bath utilizing dialysis membranes with a molecular weight cut-off of 35 kDa (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA). 24 hours prior to experimentation, dialysis membranes were immersed in distilled water. At the time of experimentation, 2 mL of freshly prepared GEM-SNEDDS (containing 2 mg GEM) and 2 mL GEM dispersion (containing 2 mg GEM in ethanol) were sealed in dialysis membranes and suspended in 30 mL of simulated intestinal fluid (SIF i.e. PBS, pH 7.4) and simulated gastric fluid (SGF, pH 1.2) as release medium maintained at 37±0.5°C at 100 rpm. Aliquots (1 mL) were withdrawn periodically at fixed time intervals (0 hr, 2 hr, 4 hr, 8 hr, 12 hr, 16 hr, 20 hr, and 24 hr) and were replaced with equal volume (1 mL) of fresh release medium to maintain constant volume. Aliquots were filtered with 0.45 µm PTFE membrane filters and the cumulative amount of drug released was analyzed by UV-spectrophotometer at 268 nm. The sensitivity and accuracy of the UV-spectrophotometer were verified at the time of development of the calibration curve. Each formulation was analyzed in triplicate (Thota et al. 2023, Ansari et al. 2023).

2.6. *Ex Vivo* Drug Permeation Studies

For the assessment of enhanced drug permeability of GEM-SNEDDS, *ex vivo* permeation studies using Sprague–Dawley male rats (250–300 g) were performed. The duodenal part of the intestine was cut (≈3.5 cm). To remove the excess fats, debris, mucus, and luminal contents, the intestines were washed with a normal saline solution. Freshly prepared GEM-SNEDDS were made. GEM dispersion (control) containing 1 mg/mL was prepared by mixing 2 mg (total 2mg/2mL) of the drug in a diluent (ethanol) on a magnetic stirrer. The formulations and control were injected into the duodenum with the help of a syringe while the drug leakage from both sides of the duodenal sac was avoided by tightly closing it with thread. The prepared duodenum with different formulations was placed in different chambers of the organ bath at a constant temperature of 37±0.5°C. The compartment was filled with 30 mL of PBS (pH 7.4). Aliquots of 1 mL were withdrawn at fixed times and immediately replaced with fresh medium. The samples were analyzed for drug dialyzed across the membrane with a UV-spectrophotometer and the permeability of GEM was calculated as cumulative GEM permeation across the duodenum versus time. Each sample was analyzed in triplicates and results were calculated as mean±SD (Jianxian et al. 2020, Mahmood et al. 2023).

2.7. Stability Studies

In order to evaluate the stability of the optimized GEM-loaded SNEDDS, stability studies were conducted as per ICH guidelines (Huynh-Ba and Zahn 2009). To assess the stability of the optimized GEM-SNEDDS formulations, they were added to capped glass vials and stored at ongoing conditions of 35°C±5°C, 65% relative humidity (RH), and accelerated conditions of 40°C±5°C, 75% RH. Samples were withdrawn from formulation

Table 4. Visual observations for emulsification test for multiple SNEDDS systems.

System code	Emulsion appearance	Time (s)	Grade
Group I	Rapid forming clear emulsion	14	A
Group II	Rapid forming clear emulsion	05	A
Group III	Rapid forming clear emulsion	11	A
Group IV	Rapid forming clear emulsion	15	A

Table 5. Percent (%) composition of successful nanoemulsion loaded with GEM (GEM-SNEDDS)

Formulation code	Oil (mL)	Surfactant (mL)	Co-surfactant (mL)
F1	Castor Oil (0.16 mL, 8%)	Tween 80 (1.04 mL, 52%)	PEG (0.8 mL, 40%)
F2	Olive oil (0.16 mL, 8%)	Tween 80 (1.04 mL, 52%)	PG (0.8 mL, 40%)
F3	Castor Oil (0.16 mL, 8%)	Kolliphor RH40 (1.04 mL, 52%)	PEG (0.8mL, 40%)
F4	Olive Oil (0.16 mL, 8%)	Tween 80 (1.04 mL, 52%)	PEG (0.8 mL, 40%)

after one month and analyzed for drug loading, entrapment efficiency, visual clarity, and phase separation using the UV spectrophotometric method (Shafiq un Nabi et al. 2007, Qureshi, Mallikarjun, and Kian 2015, Jianxian et al. 2020).

3. Results

3.1. Pre-Formulation Development

3.1.1. Calibration Curve

A calibration curve was plotted using the concentrations made for working standards (figure 2). The calibration curve was later on utilized to find the unknown concentration of GEM in different formulations for solubility studies, entrapment efficiency, and loading content. The value for R^2 obtained was 0.9966.

3.1.2. Solubility and Compatibility Studies of GEM

The solubility of GEM was assessed in different components of the system i.e. oils, surfactants, and cosurfactants (figure 3). Among the oils selected, GEM showed the highest solubility in olive oil (0.62±0.45 mg/mL) and castor oil (0.712±0.61 mg/mL). Amongst various surfactants investigated, GEM exhibited higher solubility in Tween 80 (0.4583±0.397mg/mL) as compared to Kolliphor RH40 (0.2937±1.050 mg/mL). Among co-surfactants, PEG-400 exhibited maximum solubility (0.298±1.002 mg/mL) as compared to PG (0.493±0.996 mg/mL). PEG-400 and PG were used for further study based on their use in previous studies. The solubility of different components of SNEDDS formulations is combined in Table 2.

3.1.3. Selection of Excipients Based on Solubility Studies of GEM

A SNEDDS formulation with self-emulsifying properties can result in the formation of drug agglomerates or drug precipitation in the lumen of the gut. Therefore, the selection of appropriate excipients is compulsory. Such excipients are chosen based on hydrophilic lipophilic balance (HLB) values that upon dilution can retain the drug in solubilized form. Excipients were selected on the basis of compatibility, and those with the best emulsifying properties suitable for GEM were selected. olive oil and castor oil were chosen to be oil phases, Tween 80 and Kolliphor RH40 to be surfactants, and PEG-400 and PG to be co-

Table 6. % Entrapment and drug loading of optimized formulations of GEM-SNEDDS.

Formulation code	Entrapment efficiency (%)	Drug loading (%)
F1	99.89±0.0004	3.36±0.38
F2	99.91±0.0012	3.14±0.028
F3	99.96±0.0045	2.5±0.001

Table 7. Particle size, ζ potential and PDI of 2 mL optimized GEM-SNEDDS at room temperature.

Formulation code	Particle size (±σ) (nm)	PDI	ζ potential (±σ) (mV)	Conductivity (mS/cm)
F1	182.5±84.39	0.190	-5.52±9.87	0.0988±0.013
F2	152.5±87.13	0.451	-16.8±3.6	0.0418±0.005
F3	**94.81±82.73**	**0.406**	**-12.1±6.83**	**0.0207±0.008**
F4	412.0±65.24	0.261	-2.98±4.47	0.0252±0.054

surfactants. The compatibility between selected surfactants, co-surfactants, and oils was studied to choose the best components of the systems. All selected excipients resulted in clear and homogenous nano-emulsions in combinations described previously (section 2). These combinations were then further mixed in different weight ratios ranging from 1:1 to 1:9. Ratios of 8:52:40 for oil:surfactant:co-surfactant resulted in a larger emulsification region, as shown in Table 3. The nano-emulsion region narrows down as the ratios of Smix decrease. These systems were observed visually for emulsification region and characterized according to the grading system described in section 2.2.4.

3.1.4. Assortment of Excipients Based on Self-Nano-Emulsification Efficiency

At ambient temperature, a self-emulsification system must exhibit clear and homogenous nano-emulsions or liquid (Mahmood et al. 2023). The *in vitro* performance of selected systems was visually assessed based on the grading system mentioned above and results are presented in Table 4. Systems were used without the addition of water to evaluate any phase separation upon dilution. Visual observations showed that all selected SNEDDS systems were of grade A. The results well agreed with some previous studies (Jianxian et al. 2020).

3.2. Development of GEM-loaded SNEDDS

Based on the results of previous steps, the composition of finally selected systems into which 2 mg of GEM was incorporated, is presented in table 5. The process of preparing SNEDDS concentrates was by simple admixture of the excipients. This ease of preparation supports the core theme of the manufacturing of SNEDDS at an industrial scale i.e. scale-up manufacturing.

3.3. Encapsulation Efficiency and Drug Content Analysis

All the formulations showed encapsulation of more than 95%. Due to the nature of GEM, a maximum drug content of 3.36±0.38% was observed. The entrapment efficiencies and drug contents of selected and optimized formulations are presented in Table 6.

3.4. Particle Size, Zeta-Potential, and PDI Analysis

Droplet size, ζ potential, and PDI of optimized SNEDDS formulations are presented in Table 8.

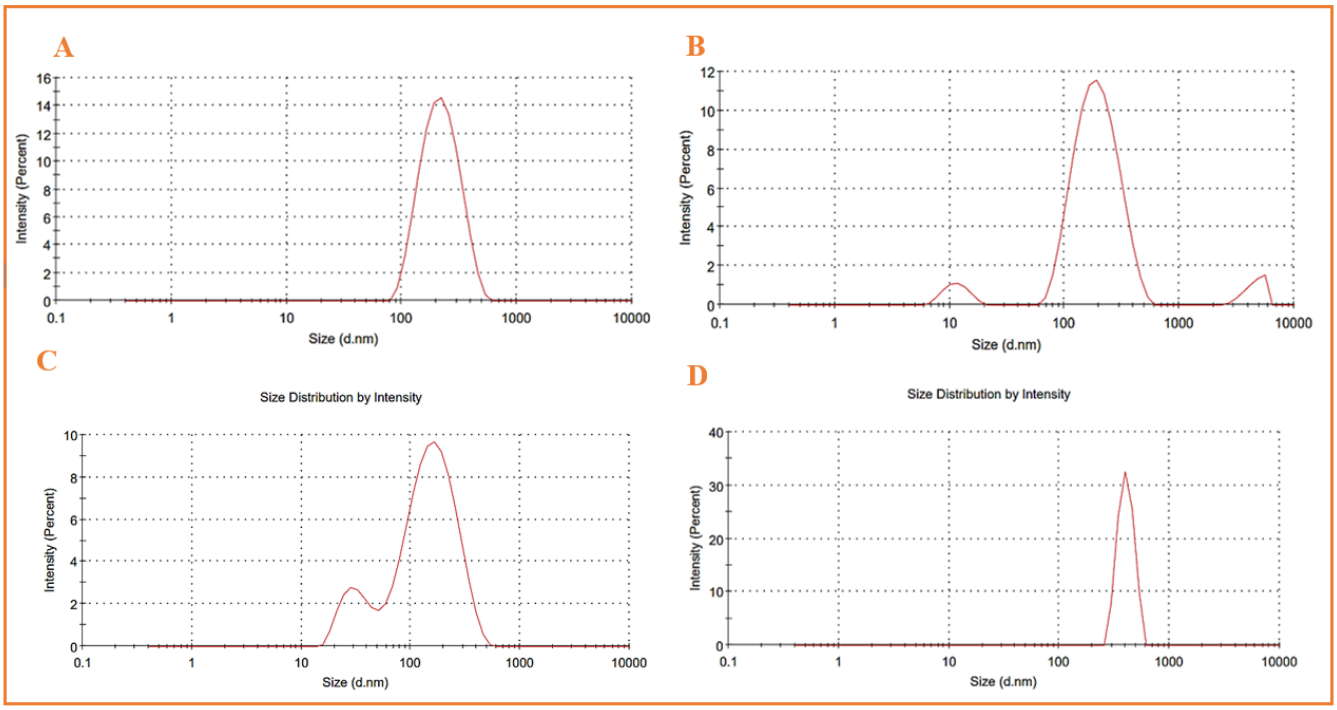

Figure 4. Zeta potential of GEM-SNEDDS conducted at room temperature. A-F1, B-F2, C-F3, D-F-4.

All GEM-SNEDDS formulations showed negative ζ potential values within the acceptable range. The ζ potentials of GEM-SNEDDS were found to range between -16.8 mV and -2.98 mV. The average globule size ranged between 94.81 nm and 412 nm. For F1 and F3, it was 182.5 nm and 94.81 nm respectively. The ζ potential of F1 and F3 was -5.52±9.87 mV and -12.1±6.83 mV respectively (figure 4, table 7).

3.5. In Vitro Drug Release Study

The *in vitro* release profile of different GEM-SNEDDS formulations in SGF and SIF along with pure GEM suspension (as a control in ethanol) is presented in Figures 5 and 6. The release profile signifies that GEM release from SNEDDS formulations produced a continuously delayed drug release in PBS (pH 7.4) and HCl (pH 1.2) as compared to pure GEM suspension. Within the initial 2 hours of the *in vitro* release study, 79.8% of pure GEM was released in SIF showing burst release while no significant amount of GEM was released from all GEM-SNEDDS. Pure GEM showed a burst release of 34% release in SGF in comparison to F3 which had a release of 0.05%. F3 formulation showed delayed drug release in SGF with 100% release in 20 hours. This effect was also observed in F1 and F2 which had complete release within 16 hours. The dissolution studies were continued for 24 hours to detect the plateau phase.

3.6. Ex Vivo Drug Permeation Studies

The drug perfused through the rat's intestine is presented in Figure 7 for GEM-SNEDDS formulations and pure GEM dispersion (control in ethanol). It was found that cumulative drug diffused through rat intestines from all GEM-SNEDDS showed a delayed release effect in comparison to pure GEM dispersion which showed a burst release effect. The cumulative amount of drug diffused from F3 after 2 hours of initiation of study was 23.5% which showed a lag in release, as did all other GEM-SNEDDS formulations.

3.7. Stability Studies

The stability of SNEDDS is a significant parameter in ensuring that SNEDDS remain stable under multiple stressful and non-stressful conditions. All

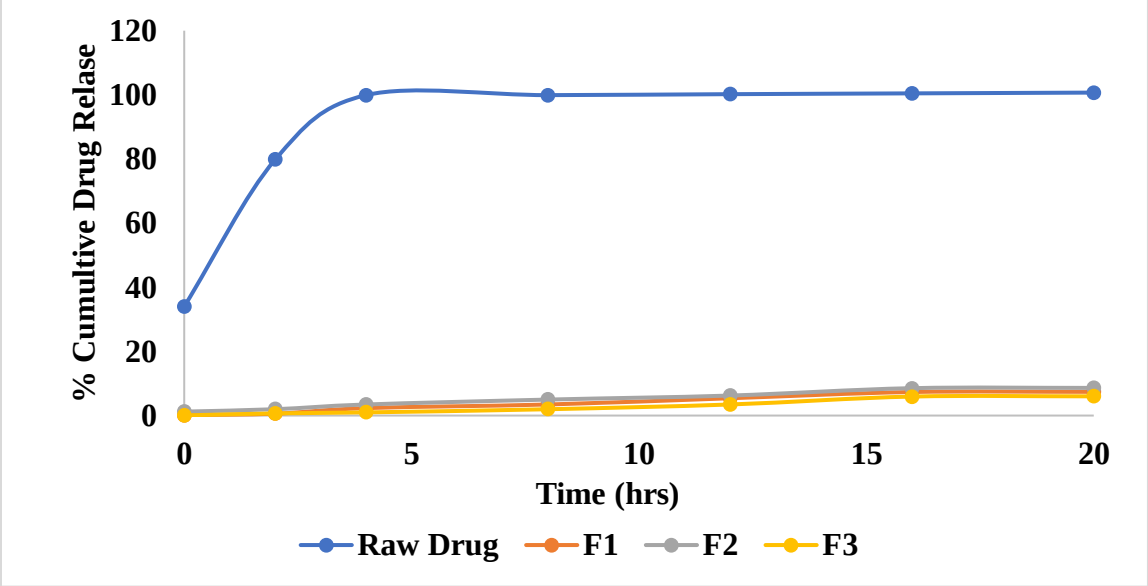

Figure 5. Dissolution profile of GEM-SNEDDS formulations (F1, F2, F3) and pure GEM suspension (RD) in SGF (pH 1.2) at 37±0.5°C for 24 hr.

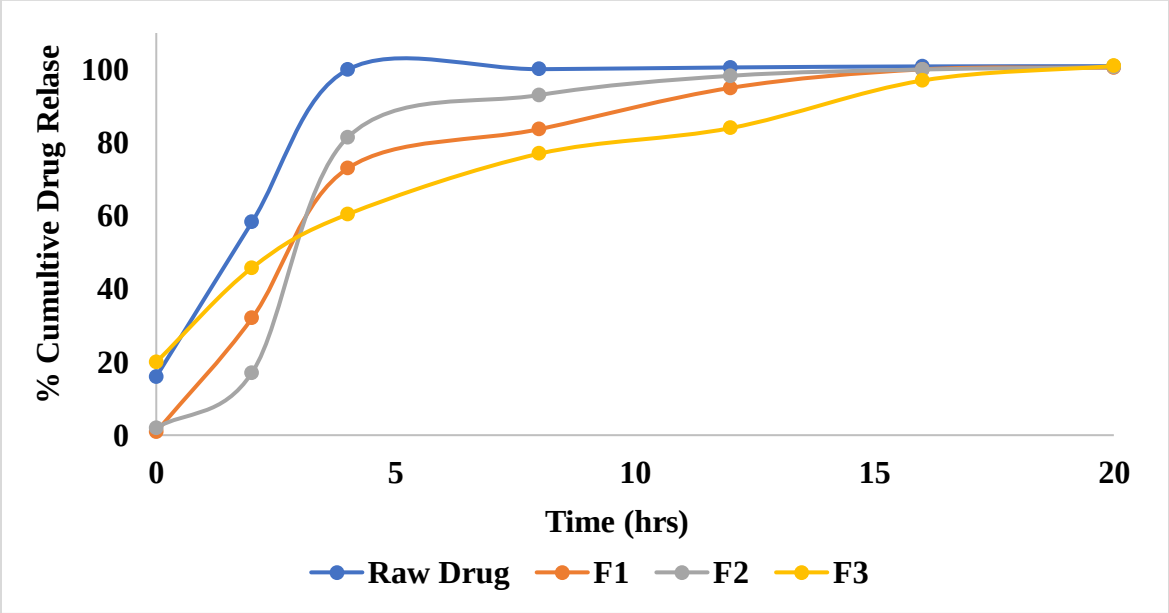

Figure 6. Dissolution Profile of GEM-SNEDDS formulations (F1, F2, F3) and GEM suspension (RD) carried out for 24 hr at 37±0.5°Cin SIF (PBS, pH 7.4).

GEM-SNEDDS showed no signs of phase separation, or any instability issues when assessed visually. For ongoing stability testing, F3 had %EE decreased from 99.96% to 99.47%. Similarly, for accelerated stability testing, F3 had %EE dropped to 9.82%. This showed that F3 retained its drug content with only minute changes and it was stable for a period of one month (table 8).

4. Discussion

GEM, a nucleoside analog, is used as the preferred drug of choice for the treatment of non-small lung, pancreatic, bladder, and breast cancers. A

significant limitation of GEM is its short half-life (2-6 hours) due to rapid metabolism and fast renal clearance, therefore necessitating high doses leading to severe side effects (renal and hematological toxicities) (Dorjee and Long 2018). On the other hand, it belongs to BCS class III that's why its GIT permeability is low. Perhaps that is why no oral formulation of GEM is present in the market (Affram et al. 2020).

The composition of SNEDDS includes an admixture of different ratios of surfactant:co-surfactants (Smix) and adding them to oils. Surfactants are necessary as they create a thin film around each nano-emulsion particle, reducing interfacial tension and providing a barrier to coalescence, thus avoiding aggregation of GEM inside GI lumen. Most importantly, negative interfacial tension and film required for creating a repulsion force inter-particle, are barely accomplished with the use of a single surfactant, hence necessitating the presence of a second component of SNEDDS, the co-surfactants. In SNEDDS, co-surfactants determine the droplet size, and thermodynamic stability and, hence stabilize the SNEDDS (Ijaz et al. 2016). Co-surfactants decline the bending stress at the interface of particles, providing elasticity for interfacial film formation in nano-emulsions for variable compositions i.e. (oil: water, water: oil, or bi-continuous). A third component, oil is a crucial part of the SNEDDS system. They work by augmenting the solubility of lipophilic drugs, increasing the portion of drug transport through the lymphatic system, and, consequently, enhancing the absorption of lipophilic drugs (Jianxian et al. 2020).

Comparing the results of compatibility studies, measured with the naked eye, showed that all excipients were compatible with each other and did not produce any phase inversion/separation or coalescence, hence they were then moved to the next step for calculating their capacity for solubilizing GEM based upon which selection of SNEDDS excipients was made, consistent with previously conducted similar studies (Ashfaq et al. 2022).

The selection of components/excipients is crucial for the preparation of SNEDDS. The drug content of GEM rests upon the solubility of GEM in multiple excipients of SNEDDS. Selection of components/excipients is based on solubility data which then helps in selecting suitable oil, surfactant, and co-surfactant having maximum solubilizing capacity for GEM attaining maximum drug content/loading (Jianxian et al. 2020). Comparing the results for the choice of excipients, it appeared that the self-nano-emulsifying properties of systems with surfactant Kolliphor RH40 were better compared to Tween 80. Nevertheless, the solubility of GEM in Kolliphor RH40 and Tween 80 was higher compared to all others. Kolliphor RH40 being a hydrophilic surfactant aids in inducing self-emulsification of oil phase in aqueous media (Eleftheriadis et al. 2019). Tween 80 acts as a drug carrier (Dannenfelser et al. 2004). Among the co-surfactants, PG and PEG-400 displayed more solubility and, thence, were selected. Among multiple oils, castor oil and olive oil were selected because of their highest solubility for GEM. Olive oil is reported to improve hypercholesterolemia because of its ability to diminish blood cholesterol (Balata et al. 2016). Castor oil possesses long-chain fatty acids. These fatty acids, made up of more than 12 carbon atoms, are able to direct the formed nano-emulsion to the lymphatic system where they bypass hepatic metabolism (Sharma et al. 2011). Furthermore, the visual clarity of formed systems may be due to increasing concentration of surfactant, low concentration of oil, and co-surfactant. The surfactants might have lessened oil concentration at the interface and, therefore, reduced the size of the formed emulsion as reflected by visual transparency. Comparable findings were also reported in other research conducted previously (Jianxian et al. 2020).

We tested the self-nano-emulsification effectiveness of SNEDDS systems by

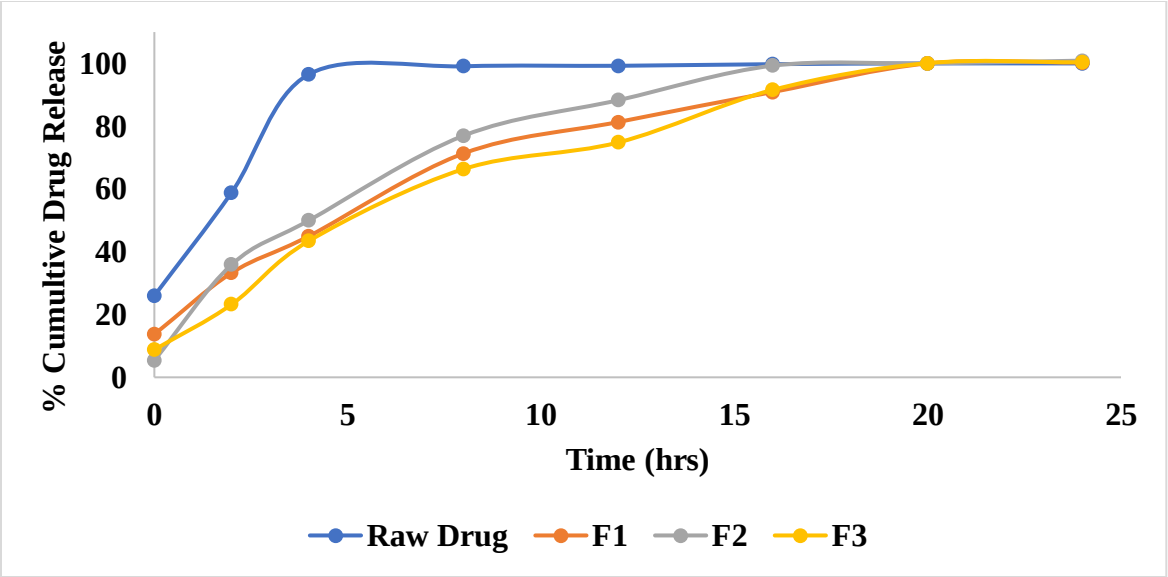

Figure 7. Ex vivo diffusion study carried for 24 hours for reconstituted GEM-SNEDDS formulations (F1, F2, F3, F4) and pure GEM suspension (C) in PBS, pH 7.4 at 37±0.5°C using rat intestine.

emulsification time and/or rate of emulsification. Emulsification is considered to be rate rate-limiting step in drug absorption. The results presented in Table 5 showed that short self-nano-emulsification time indicated their ability for easy and spontaneous emulsification upon interaction with GI fluids. The results also described that self-nano-emulsification is reliant on upon composition of the SNEDDS system and ratios in which oil:surfactant:co-surfactant are used. Results showed that the self-nano-emulsification process for the prepared SNEDDS system was spontaneous and reduced the time for self-emulsification when surfactant concentration increased. Owing to this behavior, prepared SNEDDS systems might disperse quickly under controlled (mild) agitation with the aid of surfactants that have the ability to decrease interfacial tension, diffuse water into the oil phase creating interfacial disruption and, consequently, release particles in aqueous media. Consistent with previously reported research, the high concentration of surfactant with respect to co-surfactant has led to the development of a stable self-nano-emulsifying system and smaller particle size (see Table 8 for particle size distribution). An ample amount of co-surfactant made the SNEDDS system

less stable owing to its high intrinsic aqueous solubility leading to an increase in globule size (Craig et al. 1995). Many SNEDDS systems displayed phase change, a gel phase upon aqueous dilution when oil content was higher. In short, these results suggest that prepared SNEDDS systems will remain as SNEDDS upon contact with GI fluids. However, the research conducted here is qualitative and subjected to only limited interpretation. The emulsification test only provides information about the ease of dispersion or emulsification of these systems (Craig et al. 1995, Jianxian et al. 2020).

Globule size identifies the extent of GEM release and rate of GEM release and absorption. A tiny globule size permits faster dissolution and a larger surface area for absorption of GEM, which can be attained by using suitable surfactant-co-surfactant ratios. The Smix, in defined ratios, also formed a strong barrier between formed particles that barred them from coalescence. The droplet size decreased with the reduction of the oil content. When Smix: oil concentration was low, the particles formed were larger in size, which is

Table 8. Ongoing and accelerated stability testing of three GEM-SNEDDS.

Stability Testing	F1		F2		F3	
	%EE	%DL	%EE	%DL	%EE	%DL
Zero	99.89	3.36	99.91	3.14	99.96	2.57
Accelerated 40°C	99.62	3.47	99.01	3.19	99.82	2.51
Ongoing 25°C	99.87	3.38	99.42	3.17	99.47	2.47

consistent with previously reported studies (Suresh and Sharma 2011). Besides that, the presence of surfactant also presents particles for absorption and stabilizes them which further improves the thermodynamic stability of SNEDDS (Qureshi, Mallikarjun, and Kian 2015). The particle size of selected GEM-SNEDDS was 94.81±82.73 nm. The fact that droplet size did not rise upon the addition of surfactants seems beneficial from the drug delivery aspect resulting in larger surface area dispersion available for drug absorption (Hintzen et al. 2014). Another critical factor in measuring particle homogeneity is PDI (a measure of homogeneity). F3 GEM-SNEDDS had a PDI of 0.406 showing that F3 droplets were homogenous and with enhanced physical stability.

Zeta-potential suggests the stability of emulsions. Greater positive or negative ζ-potential values create a high degree of repulsion inter-particles that then create emulsion stability. The ζ-potential of optimized GEM-SNEDDS F3 was -12.1±6.83 mV making it more stable. Multiple ions of different strengths are present in the GI tract. These ions help minimize the surface charge of nano-emulsions spread from self-nano-emulsifying formulations. For this reason, SGF shielded the negative charge of SNEDDS. The ζ-potential of near to zero creates trivial repulsion between particles and leads to aggregation, confirming the larger particle formation in SGF in comparison to water. Also, the negative charge of the gastric mucus layer repels the similarly charged droplets of SNEDDS formulations, hence, shortening the gastric emptying time. Reduction in gastric emptying time results in rapid passage of GEM within SNEDDS systems from the stomach and, therefore, a diminished release of GEM is observed in the stomach. The reduced GEM release in the stomach leads to diminished gastric side effects (Jianxian et al. 2020). Negative ζ-potential also shows the presence of non-ionic surfactants attributed to the presence of fatty acids. Intestinal cells bear a negative charge because of mucosal fluid. This negative charge permits positively charged droplets a chance for better contact with GI mucosa. Thence, it is expected that at physiological pH F3 GEM-SNEDDS would reach a positive ζ-potential. It is believed that high ζ-potential (negative or positive) particles do not coalesce due to electrostatic repulsion, but this may not hold stringently true for SNEDDS (Mahmood et al. 2023). From stability testing, it is also clear that all GEM-SNEDDS were stable, and did not have any sign of particle aggregation or phase separation.

The release of GEM *in vitro* and *ex vivo* studies can be credited to the formation of nano-emulsion. This dissolution of GEM from GEM-SNEDDS can lead to enhancing absorption and boosting the bioavailability of GEM. A high relationship between droplet size and release of GEM was also observed. F3 GEM-SNEDDS with the least droplet size showed delayed GEM release in comparison to all other SNEDDS as well as pure GEM dispersion. This may be associated with a larger surface area by droplets for dissolution and permeability of GEM. Among all GEM-SNEDDS, F3 had the highest GEM release, preventing release in SIF, bypassing the gastric environment, and carrying the cargo (GEM) to the intestine for

maximum absorption. The better release profile of GEM-SNEDDS in SIF specifies that the GEM will release efficiently from SNEDDS in the intestine (Mahmood et al. 2023, Jianxian et al. 2020). The burst release obtained for pure GEM is undesirable as it shortens the overall duration of the drug's therapeutic effect (Yoo and Won 2020). The results from stability testing for all selected GEM-SNEDDS displayed good stability under ongoing and accelerated stability conditions. There were only minute changes observed in %EE and %DL of all GEM-SNEDDS. Due to the suitable concentration of oil and Smix, the phase change/separation and or any other instability issues were not observed (crystallization, flocculation, phase inversion).

5. Conclusion

Our study suggests that GEM-SNEDDS prepared with castor oil, Kolliphor RH40, and PEG-200 showed superior results to all other combinations tested. For zeta-potential, PDI, and size distribution, F3 showed the best results, which was also evident from *in vitro* release and *ex vivo* permeation studies. F3 was stable and was the most stable GEM-SNEDDS. Owing to these results, we conclude that the permeability of GEM has been enhanced when incorporated into SNEDDS, which in turn will ultimately improve its bioavailability. The enhanced release till 20 hours decreases the frequency of drug administration in patients treated with GEM. GEM being studied in clinical trials as a first-line agent for breast cancer, will have a decreased frequency of administration. This will ultimately increase patient compliance. The side effects of GEM can be reduced or avoided when delivered through SNEDDS.

Conflict of Interest

The authors declare that they have no conflicts of interest to disclose.

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Study Approval

There are no human subjects involved so, this study requires no institutional or ethical review board approval.

Consent Forms

NA.

Authors Contributions

SG was responsible for conceptualization, methodology, validation, formal analysis, investigation, writing original draft, and visualization. SAA was responsible for conceptualization, methodology, investigation, writing – review & editing. KS and UI were responsible for conceptualization, methodology, supervision, writing – review & editing, and project administration.

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