In vitro and *ex vivo* Studies of Linagliptin Loaded Non-Ionic Surfactant Vesicles Using Statistical Optimization

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Original Research Article

ABSTRACT

Aim: The aim of this work was to increase the bioavailability of linagliptin, a BCS class-III drug, by improving permeability. For this purpose, linagliptin loaded different non-ionic surfactant vesicles were formulated and evaluated using statistical optimization.

Methods: Two independent variables selected were surfactant span 60 (X_1), cholesterol (X_2) and three dependent variables were evaluated like percent drug entrapment efficiency (Y_1), percent drug content (Y_2) and percent cumulative drug release (Y_3) respectively. Based on the central composite design of user-defined design, nine batches of non-ionic surfactant vesicles (Niosomes) were prepared by thin film hydration method (TFHM) and modified ether injection method (MEIM) each respectively. The relation between the dependent and independent variables was drawn out from the mathematical equation and response surface methodology (RSM). Statistical analysis was performed using ANOVA.

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Results: Microscopic observation confirmed that all particles were uniform in size and shape. Particle size of non-ionic surfactant vesicles measured by SEM was between 10µm to 100µm that given the evidence of large unilamellar vesicles formed by TFHM. *In vitro* dissolution studies were carried out in phosphate buffer (pH 7.4) for 8 hours according to the USP paddle method. The maximum and minimum drug releases were observed as 85.5% and 79.65% from non-ionic surfactant vesicles respectively, after 8 hours. Release kinetics was studied in different mathematical release models to find out the linear relationship and release rate of the drug. The FTIR studies have been done to confirm no interaction along with drug and polymer. In this experiment, it is difficult to explain the exact mechanism of drug release. But the drug might be released by fickian diffusion as the correlation coefficient (R^2) best fitted with zero order and release exponent (*n*) was less than 0.43.
Conclusion: At last it can be concluded that all *in vitro* and *ex vivo* experiments exhibited promising result to treat type II diabetes mellitus with linagliptin loaded non-ionic surfactant vesicles.

Keywords: Non-ionic surfactant vesicles; linagliptin; EIM; TFHM; factorial design.

1. INTRODUCTION

The top 50 drugs selling in the world have 84% oral delivery [1]. Variety of approaches have been tried to enhance the oral bioavailability of drugs using the excipients with approved or GRAS (generally regarded as safe) status. Micronization by spray-drying, freeze-drying, crystallization, and milling; nanosizing into nanoparticles by various techniques, microemulsions, self-emulsified and self-microemulsified drug delivery systems, microspheres, niosomes, liposomes etc. have been widely researched for enhancement of oral bioavailability [2, 3]. Much effort has been going on to develop sophisticated drug delivery systems such as non-ionic surfactant vesicles (niosomes) for oral application. Niosomes are non-ionic surfactant vesicles having a lamellar structure formed by self-assembly of surfactant molecules. Nonionic surfactant vesicles have been used to deliver a number of drugs and have shown pronounced benefits of enhanced bioavailability, sustained release, targeted delivery, decreased side effects, high stability and easy modification [4, 5]. Niosomes can entrap both hydrophilic and lipophilic drugs, either in an aqueous layer or in a vesicular membrane made of lipid material.

Diabetes is a group of chronic carbohydrate metabolism disorders resulting from the diminished or absent action of insulin by altered secretion, decreased insulin efficacy or combination of both the factors leading to hyperglycemia. Researchers attempted for years to enhance the oral bioavailability, sustain the drug release for better patient compliance and reduced side effects of the most potent, selective and orally active hypoglycemic linagliptin. The conventional dosage form of linagliptin i.e. tablet has been modified by various approaches to get the desired results. Linagliptin is an oral anti-diabetic drug used in the treatment of type II diabetes mellitus which acts by inhibiting the enzyme dipeptidyl peptidase-4 (DPP-4) [6, 7]. It belongs to BCS class-III drug i.e., it has high solubility and low permeability and has a low bioavailability of 30%.

Therefore the present research work is aimed to develop linagliptin loaded nonionic surfactant vesicles to achieve a sustained release profile with maximum encapsulation efficiency by using different polymers thus as increase bioavailability. Focus on niosomes was given since particles in this drug delivery system improve permeability of drug as well as bioavailability.

2. MATERIALS AND METHODS

2.1 Materials

Linagliptin was gifted sample from Eskayef Bangladesh Limited. Span-60 (MERCK, Germany), Cholesterol (ALFA Aesar, UK), Diethyl ether (MERCK, Germany), Methanol (MERCK, Germany), Propanol-1 (MERCK, Germany) etc. are all laboratory analytical grade.

2.2 Nonionic Surfactant Vesicles Preparation

For the preparation of linagliptin loaded nonionic surfactant vesicles using central composite design, user-defined factorial design was adopted to optimize the formulation parameters and to study the influence of independent

formulation variables on dependent variables. Design Expert® software (Trial Version 7.1.6, Stat-Ease Inc., MN) was employed for this purpose. Nine experimental runs were designed by selecting two parameters (span-20 and cholesterol amount) at three levels each (low, medium and high) that is shown in Table 1. The amount of drug (50 mg) was kept constant for each batch. Percent Drug Entrapment Efficiency (Y_1), Percent Drug Content (Y_2) and Percent *in vitro* release (Y_3) were selected as dependent variables. The results obtained for each response were fitted to a quadratic polynomial model explained by a nonlinear equation:

$$Y=\beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_1X_2 + \beta_4X_1^2 + \beta_5X_2^2$$

Where Y is the measured response, $\beta_0 – \beta_5$ are regression coefficients and X_1 and X_2 are independent factors. The models were validated by analysis of variance (ANOVA) and multiple correlation coefficient (R^2) tests.

2.2.1 Preparation of linagliptin niosomes by ether injection method (EIM)

Cholesterol (CHO) and surfactant were dissolved in 8 ml diethyl ether mixed with 2 ml methanol containing a weighed quantity of linagliptin. The resulting solution was slowly injected using a micro syringe at a rate of 1ml/min into 20 ml of hydrating solution phosphate buffer (pH 7.4). The solution was stirred continuously on a magnetic stirrer and the temperature was maintained at 60-65ºC. As the lipid solution was injected slowly into the aqueous phase, the differences in temperature between phases cause rapid vaporization of ether, resulting in spontaneous vesiculation and formation of niosomes, which is shown in Fig. 1 and Table 2. A 2^3 (two factors, three levels) central composite design was employed to study the effect of independent variables on dependable variables. All the formulations as per experimental design were prepared using similar procedure by addition of various quantities of surfactant and cholesterol.

2.2.2 Preparation of linagliptin niosomes by thin film hydration method (TFHM)

In this method surfactant (span 60) and cholesterol were dissolved in 8ml diethyl ether. A weighed quantity of drug was dissolved in 2ml methanol. Then the two solutions were mixed together in a round bottom flask. Using the rotary flash evaporator, the organic solvents were removed at room temperature of 20ºc. The flask was rotated at 135 rpm which leaves a thin layer of solid mixture on the wall of the flask that is shown in Fig. 1. The dried film is then rehydrated with 20 ml phosphate buffer (pH 7.4) solution at the temperature of 60-65ºC for a specified period of time (about 3 hours) with gentle agitation. Finally, the niosomal dispersion was stabilized by keeping at 2-8^{0}C for 24 hours. All the formulations as per experimental design were prepared using similar procedure by addition of various quantities of surfactant and cholesterol.

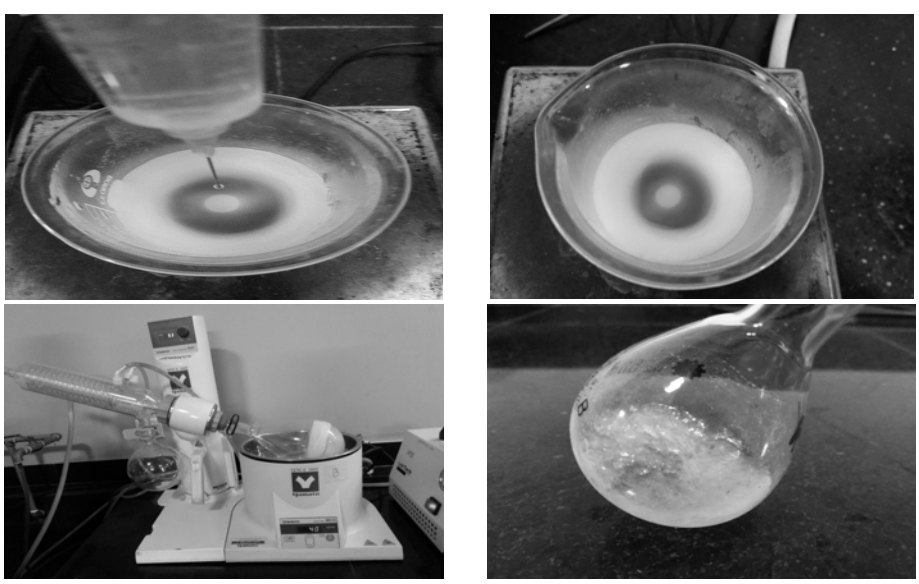


Fig. 1. Preparation of Niosomes by a) Ether Injection Method (EIM) and b) Thin Film Hydration Method (TFHM) Respectively

Table 1. Independent variables and their levels in experimental design

Independent variables	Levels (Actual coded)		
	Low (-1)	Medium (0)	High (+1)
X_1: Span-60 (mg)	50	100	150
X_2: Cholesterol (mg)	50	100	150
Dependent variables		**Goals**	
Y_1: Drug Entrapment Efficiency (%)		Maximize	
Y_2: Drug Content (%)		Maximize	
Y_3: CDR at 8 hrs (%)		Minimize	

Table 2. Design layout of experiments as per user defined factorial design

Run	Drug (mg)	Coded value		Actual value (mg)	
		Span 60	Cholesterol	Span 60	Cholesterol
R1	50	-1	-1	50	50
R2	50	0	-1	100	50
R3	50	+1	-1	150	50
R4	50	-1	0	50	100
R5	50	0	0	100	100
R6	50	+1	0	150	100
R7	50	-1	+1	50	150
R8	50	0	+1	100	150
R9	50	+1	+1	150	150

2.3 Evaluation of Linagliptin Loaded Niosomes

2.3.1 Determination of percentage of drug encapsulated in the niosomes

Entrapment efficiency was measured by measuring the un-entrapped free drug. The free drug was determined by subjecting the niosomal formulation to centrifugation at 4000 rpm for 2 hrs to separate the free drug. After centrifugation, the supernatant was collected. The collected supernatant was analyzed for the drug content spectrophotometrically at 235 nm. The percent entrapment was determined by following formula:

$$\%\ \text{Drug Entrapment efficiency} = \frac{\text{Amount of Entrapped Drug}}{\text{Total Amount Added}} \text{X } 1oo$$

2.3.2 Drug content

Drug content was determined by disrupting the niosomal formulation by propane-1-ol, diluted suitably using phosphate buffer pH 7.4 and analyzed for the drug content spectro-photometrically at 235 nm. The percentage of drug content was calculated by using the following formula:

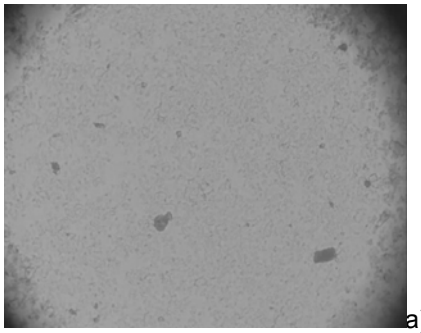
$$\%\ \text{Drug content} = \frac{\text{Sample absorbance}}{\text{Standard absorbance}} \text{X } \frac{\text{Standard dilution}}{\text{Sample dilution}} \text{X}100$$

2.3.3 Stability studies

The stability studies of the optimized niosomal formulations were performed at different conditions of temperature and the effect on physical characteristics and drug content was noted. The niosomal dispersions were kept in the airtight containers and stored at refrigeration temperature (2-8°C) and at room temperature (30 ± 2°C) for 21 days and the 1.0 ml samples were withdrawn on different days (7, 14 and 21). The stability of the formulation was analyzed by measuring entrapment efficiency and drug content.

2.3.4 Morphological characterization of niosomes

The vesicle formation by the particular procedure was confirmed by optical microscopy in 45 X resolution. The niosomal suspension placed over a glass slide and fixed over by drying at room temperature, the dry thin film and ether injection of niosome suspension observed for the formation of vesicles. The microphotography of the niosomes also obtained from the microscope by using a digital camera (Fig. 2). The detailed surface characteristic of the selected linagliptin niosomes formulation was observed using a scanning electron microscope.


a)

b)

Fig. 2. Particle size Observation by Optical Microscopy of Linagliptin Niosomes a) LEIM and b) LTFHM Respectively

2.4 Particle Size by Scanning Electron Microscopy (SEM)

Vesicle size of selected niosomal dispersion was determined by an optical microscope and vesicle size, shape and surface property of the selected formula was studied using a scanning electron microscope.

2.5 Drug-Excipient Compatibility Study by FTIR Spectroscopy

Drug-excipient compatibility studies were done in order to evaluate any interaction between drug and polymers used in the preparation of niosomes. FTIR spectroscopy was carried out to check the compatibility between linagliptin and surfactants used.

2.6 Permeability or Diffusion Studies

2.6.1 *Ex vivo* permeability study using chicken intestinal sac

Ex vivo permeability study was carried out by using chicken intestinal sacs, niosomal suspension and phosphate buffer (pH 7.4) which was used as dissolution media. Dissolution studies were conducted in a dissolution apparatus using USP II paddle method. For isolation of everted intestine, the chicken was bought from the local market and was slaughtered. The lumen was carefully cleared from mucus by rinsing with a phosphate buffer solution (pH 7.4) and Krebs–Ringer solution. Total nine intestinal segments of six cm length were removed and transferred to oxygenated Krebs-Ringer solution. 1.5 milliliters of niosome suspension was placed in the sac which was then sealed at both ends. The sac was dipped into the receptor compartment containing the dissolution medium, 900mL of phosphate buffer (pH 7.4), was stirred continuously at 100 rpm and maintained at 37^0C. 10 ml of the sample was withdrawn at predetermined intervals from each basket, filtered with 0.45 μ filter paper and media was replenished by fresh medium. The permeability study was checked for eight hours. Fig. 3a represents the steps of *ex vivo* permeability study of linagliptin loaded niosomes using chicken intestinal sacs [8].

2.6.2 *In vitro* permeability study using cellulose dialysis tubing

In vitro permeability study was done using cellulose dialysis membrane (Specrtapor, USA) in USP II paddle method. Dialysis membrane was cut into nine (9) cm in length and soaked them in 500 ml distilled water at room temperature for 30 minutes to remove the sodium azide preserving agent. Then the membrane was rinsed thoroughly in distilled water. 1.5 ml of niosome suspension was placed in the membrane which was then sealed at both ends. The membrane was dipped into the receptor compartment containing the dissolution medium, 900mL of phosphate buffer (pH 7.4), was stirred continuously at 100 rpm and maintained at 37^0C. 10 ml of the sample was withdrawn at predetermined intervals from each basket, filtered with 0.45 μ filter paper and media was replenished by fresh medium. Absorbance was taken by using UV spectrophotometer at 235 nm. The permeability study was checked for eight hours. Fig. 3b shows the steps of *in vitro* permeability study of linagliptin loaded niosomes using cellulose dialysis membrane [8].

2.7 Dissolution Studies of Niosomes

2.7.1 Studies of dissolution profile

Absorbance values obtained from the dissolution studies were converted into percent release of

drug from the formulations of niosomes. *In vitro* dissolution studies were carried out in phosphate buffer (pH 7.4) for 8 hours according to the USP paddle method. Dissolution medium was 900 mL of phosphate buffer (pH 7.4), stirred continuously at 100 rpm and the temperature maintained at 37^0C. Finally, absorbance was taken by using UV spectrophotometer at 235 nm.

2.7.2 Release kinetics

Data obtained from *in vitro* release studies were fitted to various kinetic equations to find out the mechanism of drug release from the niosome. The kinetic models used were zero order first-order, Higuchi and Korsmeyer-Peppas to ascertain the kinetic modeling of drug release.

2.8 Successive Fractional Dissolution Time

To characterize the drug release rate in different experimental conditions, time at which twenty-five percent (T_{25}%), fifty percent (T_{50}%) and eighty percent (T_{80}%) drug released were calculated from dissolution data according to the following equations:

- $T_{25}\% = (0.25/k)^{1/n}$
- $T_{50}\% = (0.5/k)^{1/n}$
- $T_{80}\% = (0.8/k)^{1/n}$

Mean Dissolution Time (MDT) was calculated by the following equation [9]:

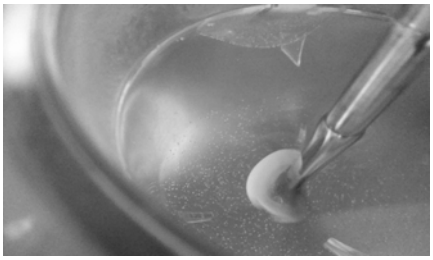
- $MDT = (n/n+1).\ K^{-1/n}$

Here, *n* is the slope and *k* is the antilog of intercept found in Korsmeyer-Peppas model. However, MDT value is used to characterize the drug release rate from the niosomes and the retarding efficiency of the surfactant and cholesterol. A higher value of MDT indicates a higher drug retaining ability of the surfactant and vice-versa.

2.9 Statistical Optimization by Factorial Design

Statistical analysis was done using ANOVA. Nine experimental runs were designed (Design Expert® software-Trial Version 7.1.6, Stat-Ease Inc., MN) by selecting two parameters (span-20 and cholesterol amount) at three levels each (low, medium and high). The regression parameters of the developed model and graphical interpretation for each response with statistical significance ($p<0.05$) were calculated by using design expert software.

Chicken intestinal membrane



a) Chicken intestinal membrane put in to dissolution vessel

Cellulose dialysis membrane

b) Dialysis sac put in to dissolution vessel

Fig. 3. Schematically presenting permeability study of Linagliptin Loaded Niosomes a) *Ex vivo*-chicken intestinal sacs and b) *In vitro*-using cellulose dialysis tubing membrane respectively

2.10 Optimization using the Desirability Function

To optimize multiple responses, they should be highly correlated with each other. In the present study, all three responses were simultaneously optimized by a desirability function that uses the numerical optimization method in the design-expert software. Recently, the desirability function approach was reported in several articles for the optimization of multiple responses. Any response that falls outside the desired limit is considered completely unacceptable. For the response to be maximized, the desirability function can be defined as:

$$d_{i,\max} = \frac{Y_i - Y_{\min}}{Y_{\max} - Y_{\min}}$$

Where, *di*,max is the individual desirability of the response to be maximized, *Yi* is the experimental result, and *Y*min and *Y*max represent the minimum and maximum possible values. If *Yi* is equal to or less than *Y* min, then *di*, max = 0; and if *Yi* is higher or equal to *Y*max, then *di*,max = 1. In order for the response to be minimized the desirability function is defined as:

$$d_{i,\min} = \frac{Y_{\max} - Y_i}{Y_{\max} - Y_{\min}}$$

Here, if *Yi* is higher than or greater than *Y*max, then *di*,min = 0; and if *Yi* is less than or below the minimum, then *di*,min = 1. After obtaining the individual desirability values for each response, the results are usually combined as a geometric mean to give a global desirable value (*D*), which is explained by equation:

$$D = (d_1 \times d_2 \times d_3 \times d_4 \times \ldots\ldots\ldots\ldots \times d_n)^{1/n} = \left(\prod_{i=1}^{n} d_i\right)^{1/n}$$

Where, *n* specifies the number of responses being optimized. According to the simultaneously assigned goals for all responses, the design-expert software determines the maximum desirability value by an extensive grid search over the domain [10].

3. RESULTS AND DISCUSSION

3.1 Studies of Percent Drug Entrapment Efficiency (DEE) and Percent Drug Content (DC) of Linagliptin Loaded Niosomes Prepared by EIM and TFHM

Different ratios formulated niosomes were analyzed for their percent drug entrapment efficiency (% DEE) and percent drug content (% DC). Results of the study were illustrated in the Fig. 4.

From the figure it can be said that percent drug entrapment efficiency of different formulations were in range of 56.2% to 89.2% whereas drug content ranging from 86.3% to 97.6% for EIM and percent drug entrapment efficiency of different formulations formed by thin film hydration method were in range of 69.4% to 91.5% whereas drug content ranging from 91.1% to 96.5%. The entrapment efficiency was found to be higher with the formulations which have high cholesterol and surfactant ratio to provide a high entrapment efficiency of linagliptin. Increase in the concentration of the surfactant leads to enhancement in the encapsulation efficiency and decrease in the leakage of the drug which might be due to the high fluidity of the vesicles but it depends upon the cholesterol amount. Minimum cholesterol content of formulations was found to cause low entrapment efficiency. This might be due to the fact that cholesterol beyond a certain level starts disrupting the regular bi-layered structure leading to the loss of drug entrapment.

3.2 *In Vitro* Drug Release Studies of Linagliptin Loaded Niosomes

In vitro release profile of linagliptin loaded niosomes given in Fig. 5 depicts the release kinetics plot of formulated niosomes.

From the Fig. 5, it has been found that the percentage release of the drug, however, increased with time for formulations LEIM1 to LEIM9 and LTFHM1 to LTFHM9. After 8 hours dissolution, the maximum drug release was found 84.11% and 85.55% whereas the minimum release was 76.74% and 79.20% for EIM and TFHM respectively. It was observed that the amount of surfactant and cholesterol affected the drug release. The increased in the surfactant (span 60) normally decreased in drug release that was seen in the formulations but a negative effect also observed in the formulations. Hence increase in the surfactant increased the drug release. The release was more controlled by increasing the cholesterol level. From the percent release of drugs, it was seen that by increasing the cholesterol ratio the drug release decreased because cholesterol acted as a retardant barrier.

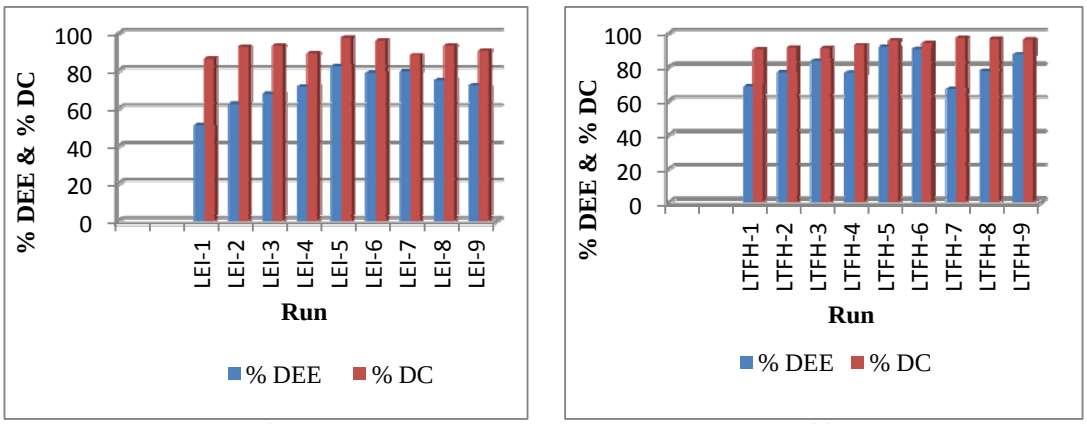
To find out the probable drug release mechanism interprets the release rate constants and R^2 values for different release kinetics of linagliptin

loaded niosomes. All the formulations were best fitted with zero order models as shown in Table 3. The data obtained were also put in Korsmeyer-Peppas models in order to find out n value, which describes the drug release mechanism. The n value of niosomes of different drug to polymer ratio was ranged less than 0.43, indicating that the mechanism of the drug release was fickian diffusion controlled.

3.3 *Ex vivo* Pemeability Studies of Linagliptin Loaded Niosomes

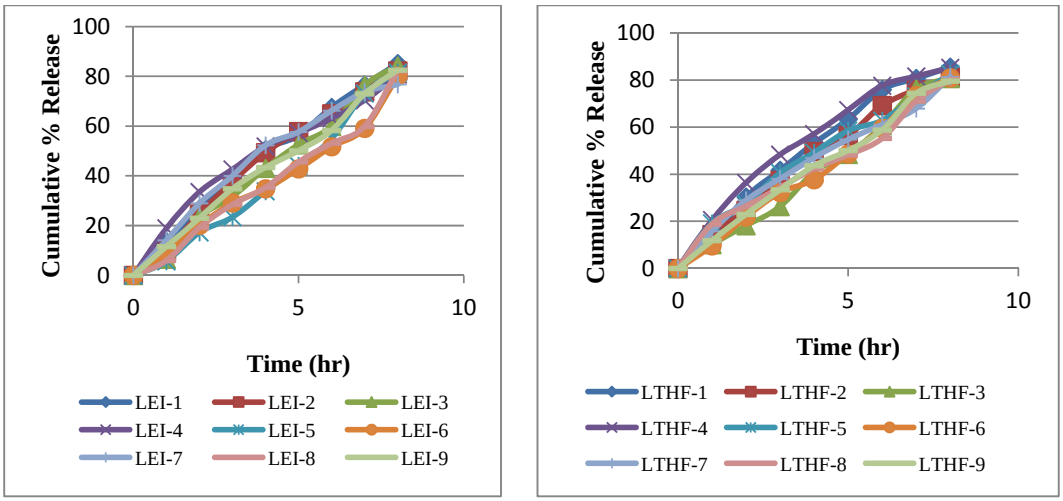
Ex vivo release profile of linagliptin loaded niosomes formed by a) EIM and b) TFHM is given in Fig. 6.

In permeability studies, the Fig. 6 indicated that the percentage drug released however increased with time. In case of ether injection method, the highest cumulative percent release was 89.01% for formulation LEIM-1 and the lowest cumulative percent release was 79.03% for LEIM-6. It was observed that the amount of surfactant and cholesterol affected the drug release profile. In case of thin film hydration method, the highest cumulative percentage release was 97.48% for LFHM-4 and the lowest cumulative percent release was 79.67% for LFH-8. However, from *ex vivo* permeability studies it is confirmed that the drug release decreased by increasing the amount of cholesterol and surfactant.



a) b)

Fig. 4. Percent drug entrapment efficiency (% DEE) and percent drug content (% DC) of Linagliptin loaded niosomes a) LEIM-1 To LEIM-9 and b) LTFH-1 To LTFH-9 respectively

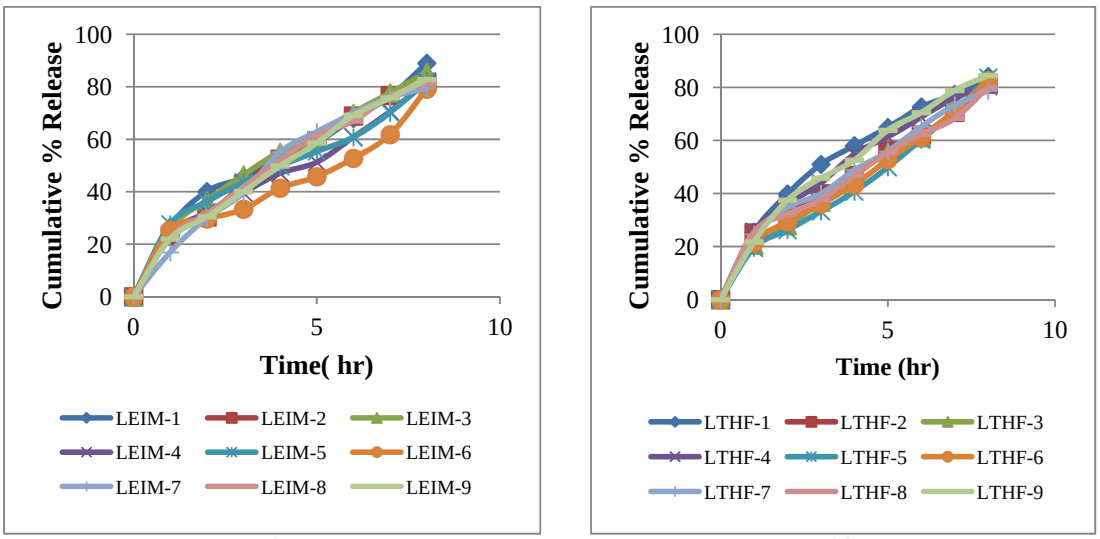


a) b)

Fig. 5. Zero order release kinetic plot of Linagliptin loaded niosomes prepared by a) EIM and b) TFHM respectively

Table 3. Interpretation of release rate constants (n) and R^2 values for different release kinetics of Linagliptin loaded niosomes

Run	Zero Order		First Order		Higuchi		Korsmeyer-Peppas	
	K_0	R^2	K_1	R^2	K_H	R^2	n	R^2
LEI-1	10.479	0.991	-0.977	0.961	31.298	0.951	0.416	0.271
LEI-2	10.35	0.987	-0.090	0.978	30.904	0.945	0.423	0.284
LEI-3	10.677	0.994	-0.095	0.929	31.104	0.908	0.411	0.293
LEI-4	9.182	0.984	-0.080	0.974	28.351	0.960	0.286	0.205
LEI-5	10.41	0.985	-0.087	0.896	29.53	0.853	0.394	0.267
LEI-6	8.572	0.913	-0.068	0.775	24.6	0.809	0.364	0.155
LEI-7	9.631	0.996	-0.081	0.967	29.402	0.970	0.375	0.247
LEI-8	9.602	0.981	-0.078	0.857	27.691	0.878	0.401	0.250
LEI-9	10.003	0.995	-0.087	0.931	29.431	0.9264	0.422	0.24
LTFH-1	10.962	0.984	-0.107	0.977	33.071	0.957	0.418	0.304
LTFH-2	10.287	0.988	-0.091	0.983	30.782	0.951	0.402	0.259
LTFH-3	10.654	0.994	-0.094	0.9176	30.636	0.8842	0.408	0.257
LTFH-4	10.421	0.994	-0.101	0.953	32.278	0.984	0.331	0.278
LTFH-5	9.6878	0.981	-0.087	0.965	29.345	0.969	0.312	0.216
LTFH-6	10.345	0.998	-0.092	0.906	29.989	0.897	0.470	0.255
LTFH-7	8.7711	0.999	-0.069	0.968	26.905	0.980	0.307	0.194
LTFH-8	9.206	0.980	-0.079	0.913	27.379	0.933	0.274	0.163
LTFH-9	9.9511	0.995	-0.085	0.947	29.303	0.928	0.416	0.240



a) b)

Fig. 6. *Ex vivo* release plot of Linagliptin loaded niosomes prepared by a) EIM and b) TFHM respectively

3.4 Comparative Study of Non-Ionic Surfactant Vesicles

A comparative release studies has shown in Fig. 7 for pure drug, marked product and formulated niosomes (LEIM-1 and LTFHM-1). At the end of 8 hours, all linagliptin loaded niosomes showed higher diffusion against pure drug and marketed products. *Ex vivo* permeability study through chicken intestinal sacs is one of the essential parts in the prediction of oral bioavailability. Besides, it can be said from the figure that linagliptin loaded niosomes by thin film hydration method (TFHM) has shown better controlled release over the period of time than the ether injection method (EIM).

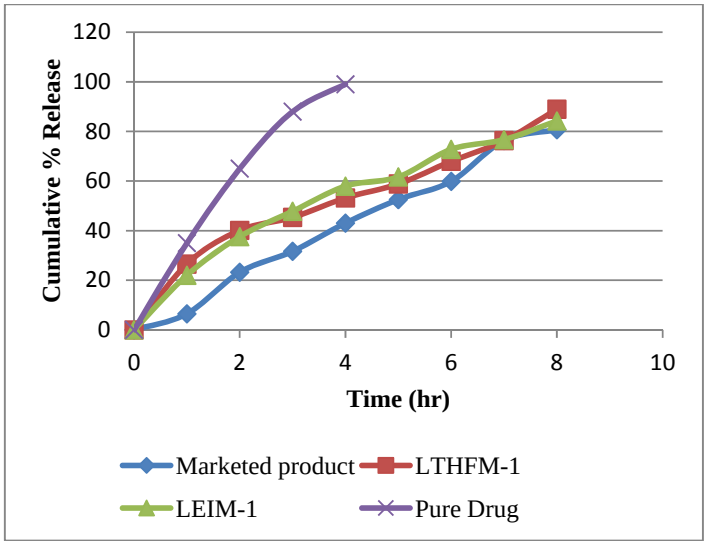


Fig. 7. Comparative Study of Linagliptin loaded niosomes

3.5 Successive Fractional Dissolution Time

Successive fractional dissolution times (hr) of linagliptin loaded niosomes of different formulations are shown in Fig.8. To characterize the drug release rate in different experimental conditions they were calculated from dissolution data. The overall results of MDT value are showing that if the amount of surfactant and cholesterol is increased the retarding affinity of formulations also increases but it happens only for a certain level. After an optimum level, increasing in the amount of surfactant and cholesterol results in decreased drug retarding affinity because after the optimum level cholesterol starts to break the bilayer of the vesicle which has to be controlled by the amount of surfactant.

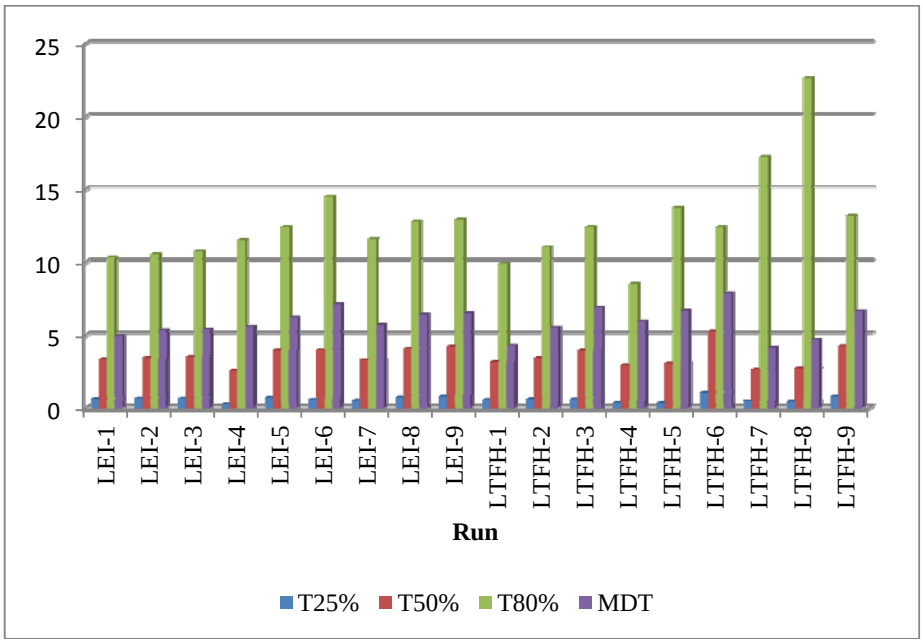


Fig. 8. Successive fractional dissolution time of Linagliptin loaded niosomes by EIM and TFHM respectively

3.6 Stability Studies

To obtain stability data of different niosomal formulations, they were kept under the different condition for several days that are given in Table 4. The intermediate stability study for LEIM-6 and LTFHM-5 was performed for 21 days according to the ICH guidelines. Drug entrapment was fixed as a physical parameter for stability testing and stability studies of selected formulation LEIM-6 and LTFH-5 showed that negligible changes in entrapment efficiency. This revealed that the formulations stabled on storage at 4±2ºC and 30±2°C RH.

3.7 Analysis of Particle Size or Vesicle Formation

Particle size of niosomal formulation at different ratio of cholesterol and surfactant was shown in Table 5. Mean particle size of the niosomal formulation was found to be in the range of 3.3 μm to 4.5 μm. It was clearly depicted from the figure that particle size of niosomal formulations was increased on increasing the cholesterol (CHO) content. CHO content provides strength to the nonpolar tail of nonionic surfactant. At low CHO content, it is to be expected that the CHO and nonionic surfactant are in close packing with increasing curvature and reducing size. As the CHO content increases, it would reduce the content of surfactants and also increased the hydrophobicity of bilayer membrane thus increasing vesicles radius in a way to establish more thermodynamic stable form. Rigid structure of bilayer membrane due to cholesterol content also provides resistance to reduce size due to sonication and results in vesicles with bigger size. In addition, it can be said from the table that linagliptin loaded niosomes by thin film hydration method (TFHM) has shown larger vesicles or particle size than ether injection method (EIM).

3.8 Fourier Transform Infrared Spectroscopy (FTIR) Study of Linagliptin Loaded Niosomes

FTIR study has done to examine drug-polymer interaction. To probe this effect FTIR study was performed on a) pure drug (linagliptin) b) span-60 c) cholesterol d) linagliptin loaded niosomes prepared by EIM e) linagliptin loaded niosomes prepared by TFHM and is shown in Fig. 9. FTIR spectrum of pure linagliptin has shown characteristic Amine N-H peaks at 3369.64 cm^{-1}. C-H peaks at 2926.01 cm^{-1} and 2850.79 cm^{-1}, C=N peaks at 1658.78 cm^{-1} and three C-N peaks at 1251.80 cm^{-1}, 1199.72 cm^{-1} and 1128.36 cm^{-1} was observed in Fig. 9(a). FTIR spectrum of formulation LEIM-3 containing span-60 surfactant has shown N-H peak at 3454.51 cm^{-1}, Imino group C=N peaks at 1639.49 cm^{-1}, one C-H peak at 2937.59 cm^{-1} as observed in Fig. 9(b). FTIR spectrum of formulation LTFH-4 has shown characteristic N-H peak at 3444.87 cm^{-1}, Imino group C=N peaks at 1639.49 cm^{-1} as found in Fig. 9(c). FTIR spectrum of surfactant span-60 has shown characteristic N-H peak at 3404.36 cm^{-1}, presence of two C-H peaks at 2918.30 cm^{-1} and 2850.79 cm^{-1}, Imino group C=N peak at 1635.93 cm^{-1}, one C-N peak at 1058.92 cm^{-1} as observed in Fig. 9(d). FTIR spectrum of cholesterol, has shown characteristic N-H peak at 3442.94cm^{-1}, presence of two C-H peaks at 2933.73 cm^{-1} and 2900.94 cm^{-1}, Imino group 1726.29 cm^{-1}, C-N peak at 1056.99 cm^{-1} as found in Fig. 9(e). The absorption peak was close to those values obtained from pure drug. These spectrums conclude that drug is compatible with polymers revealing no significant interaction between drug and polymer. Hence, FTIR report has confirmed there is no significant drug-polymer interaction so the drug is compatible with the polymers.

3.9 Scanning Electron Microscopy of Linagliptin Loaded Niosomes

The surface morphology of the niosomes was investigated by SEM. The images of (a) LEIM-3 and (b) LTFH-4 were shown in Fig. 10 to see the morphological changes that occurred due to formulation variation. Surface morphology of formulation LEIM-3 and LTFH-4 indicates that niosomal particles were appeared as discrete and round in shape with irregular surface due to the presence of the unentrapped drug. Vesicular properties of these drug carriers which formed from double layers. SEM showed the morphology of the lipids and the arrangement of the lamellar structure the encore the drug molecules.

3.10 Full Factorial Statistical Analysis

3.10.1 ANOVA tests of the quadratic model and regression analysis for the responses

The regression parameters of the developed model and graphical interpretation for each response with statistical significance were calculated by design expert software using full factorial design shown in Table 6. The relationship between the experimental variables and responses were evaluated by generating response surface plots. In the ANOVA test, the p values of the model for responses Y_1, Y_2 and Y_3

were 0.0198, 0.0135and 0.0149 for LEIM and 0.0289, 0.0220 and 0.0255 for LTFDM respectively. Thus, from the p values for this model it can be concluded that all the responses (Y_1, Y_2 and Y_3) fitted the quadratic model well ($p < 0.05$). Significance probability values (Probability > F) less than 0.05 implies that the model is significant. Moreover, in the 'lack of fit' test, which is another good statistical parameter for checking the better fitness of the model, all the responses fitted in the quadratic model by showing a non-significant lack of fit (p>0.1). In this study, the R^2 values for the responses Y_1, Y_2, and Y_3 were 93.70 %, 94.45%, and 84.02% for LEIM and 95.21 %, 96.45%, and 95.5% for LTFHM, respectively.

3.10.2 Response surface plot analysis

Three-dimensional response surface plots of the responses across the selected factors were constructed to further elucidate the relationship between the independent and dependent variables, as shown in Fig. 11. These types of plots are very useful for studying the interaction effects between two factors and for understanding how the effect of one factor will be influenced by the change in the level of another factor. As these types of plots can only express two independent variables at a time against the response, one independent variable must always be fixed.

Table 4. Stability studies of Linagliptin loaded niosomes

Run	LEI-6		LTFH-5	
Duration	**Refrigeration Temp. (4±2ºC)**	**Room Temp. (30±2°C)**	**Refrigeration Temp. (4±2ºC)**	**Room Temp. (30±2°C)**
7days	78.85	78.61	84.5	84
14 days	78.05	77.92	84.01	83.2
21days	77.69	77.03	83.75	83

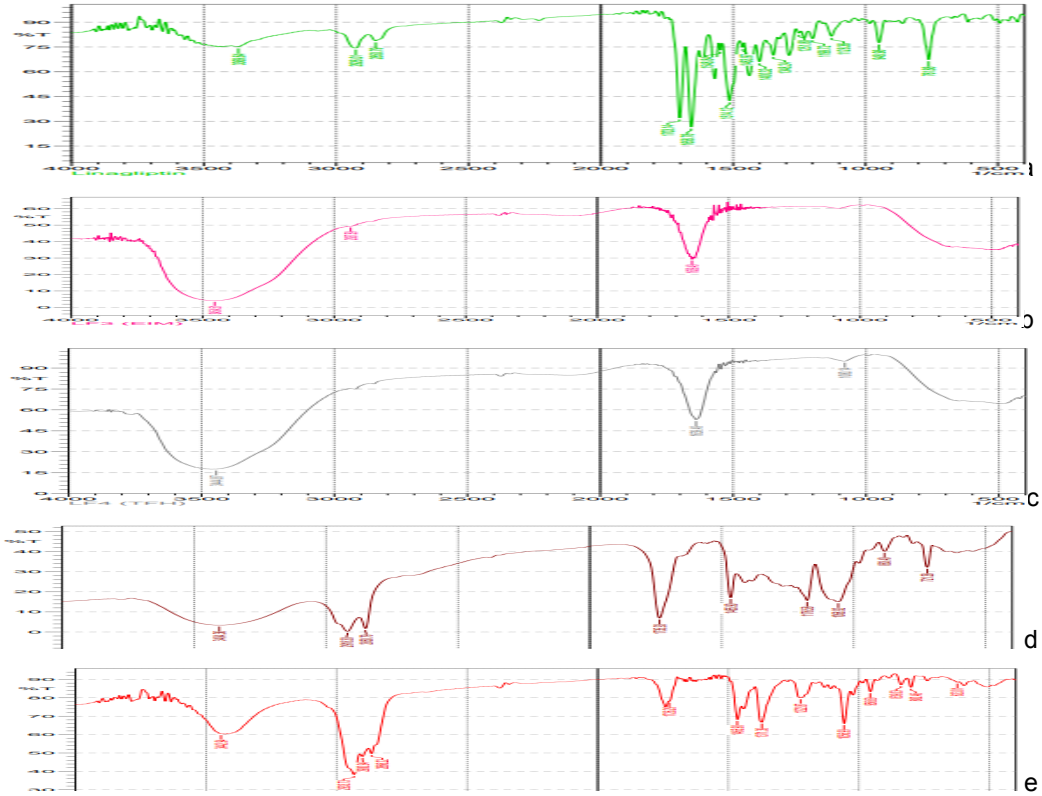


Fig. 9. FTIR Image of a) Pure Drug Linagliptin b) Linagliptin Loaded Niosomes (LEIM-3) c) Linagliptin Loaded Niosomes (TFHM) d) Span-60 e) Cholesterol Respectively

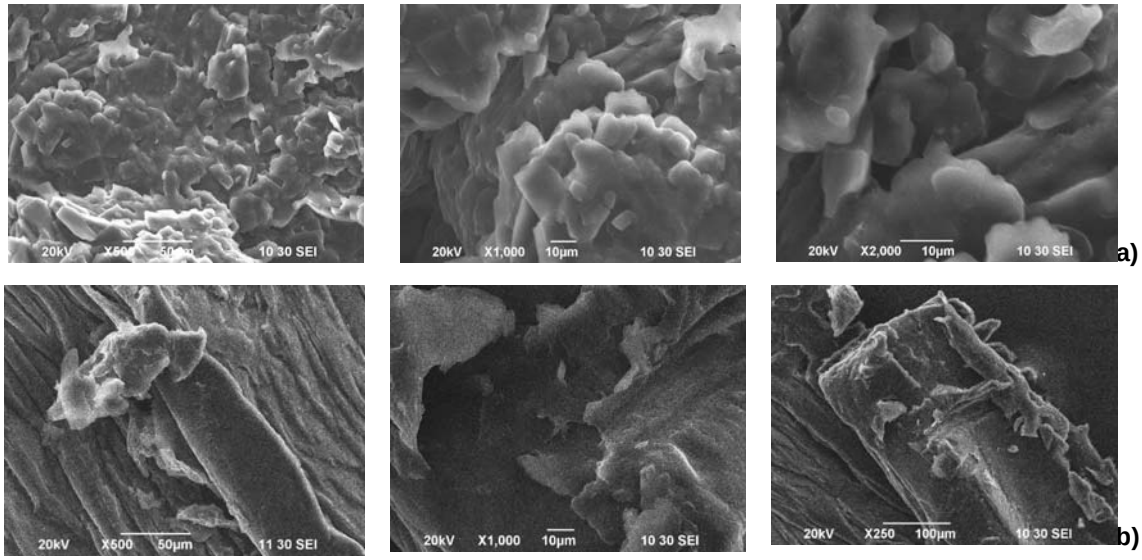


Fig.10. Scanning Electron Microscopy (SEM) of formulations a) LEIM-3 and b) LTFH-4 at different magnification respectively

Table 6. ANOVA Tests of the quadratic model and regression analysis for the responses

Method	Response	R^2	Model	Probability > F	Comment
LEIM	Y_1	0.9370	Quadratic	0.0198	Significant
	Y_2	0.9445		0.0135	
	Y_3	0.8402		0.0149	
	Lack of Fit			0.41	Not Significant
LTFHM	Y_1	0.9521	Quadratic	0.0289	
	Y_2	0.9645		0.0220	Significant
	Y_3	0.9607		0.0255	
	Lack of Fit			0.42	Not Significant

Mathematical relationship using multiple linear regressions

- **Final equation in terms of actual factors for LEIM**

Y_1 = -15.11667 + 0.53550X_1 + 1.15003X_2 - 2.43000E - 003X_1X_2 – 1.19000E - 003X_1^2 – 3.78000E - 003 X_2^2

Y_2 = + 57.2211+ 0.40497X_1+ 0.33180X_2-4.56000E-0.004X_1-1.52867E-003X_1- 1.4266E-0.003X_2

Y_3 = +93.67889 – 0.018000 X_1 – 0.21457X_2 + 6.74000E-004X_1X_2 -1.77333E-004X_1^2 +5.68667E-004X_2^2

3.10.3 Optimization using desirability function of TFHM and EIM

After studying the effects of the dependent and independent variables on the responses, the independent variables were simultaneously optimized for all three responses by using the desirability function. Responses Y_1, Y_2, and Y_3 were transformed into individual desirability shown in Table 7. Constraints were set against all of the responses. Among the responses, Y_1 and Y_2 were set to be maximized and Y_3 were set to be minimized. Equal weight and importance were given to all of the responses. Finally, the global desirability value was calculated by combining the individual desirability function as the geometric mean by an extensive grid search and feasibility search over the domain by the Design-Expert software (Stat-Ease Inc.). Fig. 12 shows the response surface plot for the desirability function holding the variable X_1, X_2.

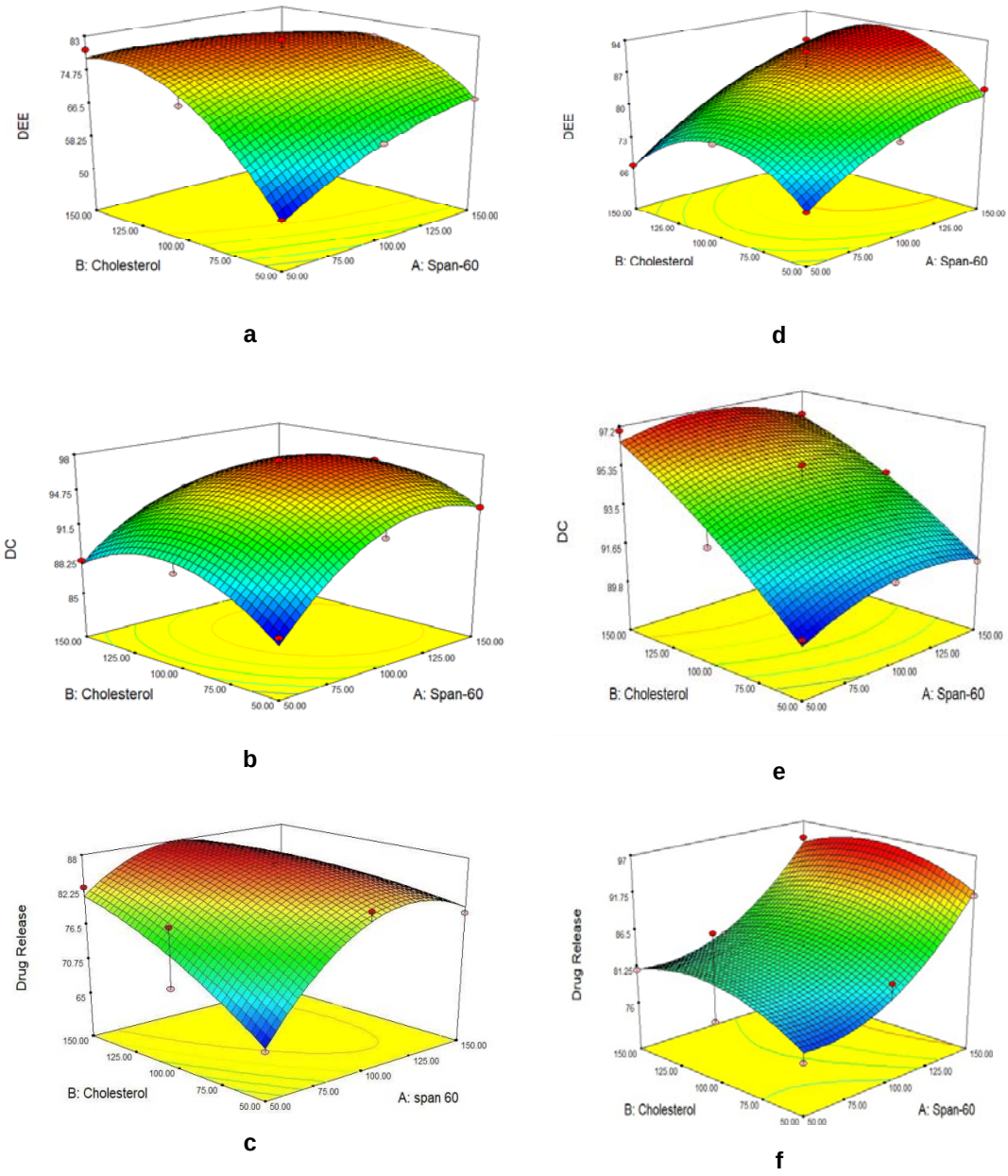
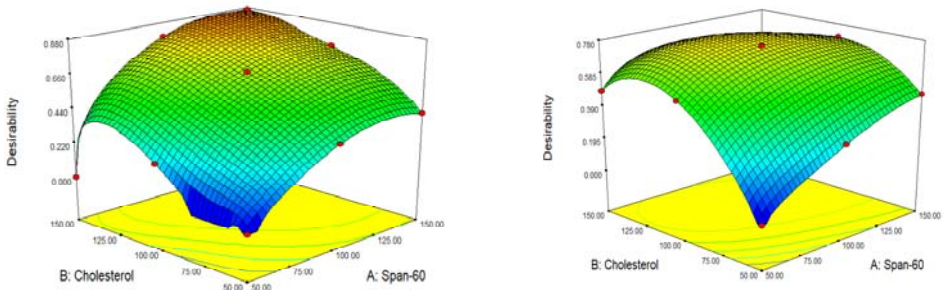


Fig. 11. Response surface showing the effects of X_1 and X_2 on the % of drug entrapment efficiency (a, d), % drug content (b, e) and % drug release (c, f) for LEIM and LFHM respectively

Table 7. Calculated values for optimized solution

Method	X1	X2	Y1	Y2	Y3	Desirability
TFHM	135.0	146.36	85.75	96.34	79.74	0.979
EIM	98.41	116.51	80.90	96.33	80.63	0.927



a) b)

Fig. 12. Response surface plot showing overall desirability (D) as a function of X_1 and X_2 for a) TFHM and b) EIM respectively

4. CONCLUSION

The present study was conducted to design linagliptin loaded controlled release non-ionic surfactant vesicles by ether injection and thin film hydration method. Ether injection method and thin flim hydration method are potentially scalable methods for producing non-ionic surfactant vesicles for delivery of hydrophobic or amphiphilic drugs. *In vitro* dissolution study showed the controlled release of drugs from the niosomes for 8 hours. From the *in vitro* dissolution data it has been established that the drug dissolution profile could be sustained by increasing the amount of surfactant and cholesterol in the formulations and where both the surfactant and span-60 are high ensured the better-controlled release. Scanning electron microscopy showed a uniform size of niosomes. FTIR data showed the absence of any new functional group and any other interaction in between drugs and surfactant. In addition, formulated niosomes can be chosen for *ex vivo* study by the chicken intestine. Non-ionic surfactant vesicles formulations containing linagliptin were successfully optimized by employing statistical tool ANOVA and response surface methodology (RSM). The results suggest that the RSM using factorial design could be a suitable approach for understanding formulation variables and for optimizing the formulation efficiently. The results further reveal that surfactant and cholesterol and its concentration can modify all the evaluation parameters significantly. Moreover, it can be said from the results that the thin film hydration method (TFHM) has shown better results in terms of all parameter than ether injection method (EIM). So, linagliptin loaded non-ionic surfactant vesicles might be a potentially controlled drug delivery system for the treatment of type-II diabetes with enhanced bioavailability by improving permeability.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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