Formulation and Evaluation of Ledipasvir Nano-suspension Through QbD Approach

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ABSTRACT: Ledipasvir, belonging to the BCS class II, is a directly acting anti-viral agent used to treat Hepatitis C virus infections. Due to poor water solubility and oral bioavailability, developing an effective delivery system for this drug has been an enormously challenging issue for the formulators. Moreover, suitable dosage forms for pediatric and geriatric patients and patients having difficulty in swallowing as well pose an added burden. Therefore, the present study aims to formulate a nanosuspension, via a solid dispersion technique, based on liquid oral suspension using the Quality by Design (QbD) method. Primarily, the compatible polymers for ledipasvir were screened using FT-IR and DSC and finally the polymers- poloxamer 188, poloxamer 407, HPC and HMPC were selected, considering their ability to turn the API into amorphous state in solid dispersions. The design of formulation and analysis with the D-Optimal design using Design Expert® Software revealed that poloxamer 188 and poloxamer 407 at 0.3:0.7 ratio of ledipasvir: polymer produced the optimized nanosuspension formulations with a statistically significant mathematical model. Subsequently, the formulations were stabilized using a suspension vehicle optimized via Box-Behnken design using the amount of xanthan gum (gm), avicel® RC-591 (gm) and citric acid monohydrate (gm) as independent variables, and viscosity (cp) & zeta potential (mv) as responses. The dissolution profiles revealed that the prepared suspensions of ledipasvir had much faster dissolution than the market products available as the tablet dosage form. *In-vivo* simulation studies using PKSolver® suggested that the absorption of the drug from the formulated suspensions was comparable to that of market product up to a single dose level (90 mg) and superseded in triplicate dose level (270 mg). The formulated suspensions were found to be stable over three- and six-month periods, as identified via accelerated stability studies. Interestingly, the dissolution profile of the stabilized suspensions was found to be similar after six months to that of the initial.

Key words: Ledipasvir, D-optimal, design expert®, Box-Behnken, PKSolver®, simulation, stability.

INTRODUCTION

A remarkable number of drug candidates exhibit insufficient drug concentrations at the absorption sites owing to poor solubility and low dissolution rate (BCS class II), and therefore low oral bioavailability.[1,2] Recently, several strategies have been found effective to improve the dissolution rate of such poorly soluble drugs where nanosuspension and solid dispersions are one of the promising techniques in this aspect.[3]

Surfactants, maintaining the drug in a dispersed state, are widely used during the formulation of dispersion, but the potential of creating toxic responses limits their usage.[4] Solubility issue of drug can be improved through micronization. The micronization technique involves the technique of colloid mills. The particle size found through this process ranges from 0.1 to 25 µm and the slightest

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portion is less than 1 µm.[5] Formulation of particles in the nano-size from micron was the subsequent task.[5,6]

The precipitation method was utilized for developing drug nanoparticles by Gassmann et al.[7] However, in this method the drug has to be soluble in at least one solvent which needs to be miscible with a non-solvent and this phenomenon limits the applicability of this method.[8] This challenge was conquered in 1995, by Muller et al. who capitalized the dispersion method for manufacturing the nanosuspension.[5] These formulators found that the drug particles of 10 to 1000 nm exhibited excellent stability in the presence of surfactants and polymers. After this milestone invention, nanosuspensions are accepted as drug carriers.[9] It is now proven that nanosizing is a promising technique to improve saturation solubility and the rate of dissolution of drugs.[10] Moreover, nanosizing of drug particles minimises drug administration doses, side effects and cost of therapy.[11]

Ledipasvir belongs to the BCS class II drug and provides its action through inhibition of the Hepatitis C virus (HCV) non-structural protein 5A (NS5A) which plays a major role in virus-related RNA reproduction and association of HCV virions.[12] Due to its poor aqueous solubility and low bioavailability, it has become a challenging task for the researchers to deliver the drug effectively. The focus of this research study was to develop a nanosuspension of ledipasvir for increasing its solubility and bioavailability.

MATERIALS AND METHODS

Chemicals and reagents. Ledipasvir (LDV) was purchased from Xiamen Halosyntech Co., LTD, China. Poloxamer 188, Poloxamer 407, povidone K17, povidone K30, potassium dihydrogen phosphate and tween 80 were collected from BASF, Germany. Hydroxypropylcellulose (Klucel™ EXF) and carboxymethylcellulose sodium 7MF (Aqualon® CMC 7MF) were obtained from Ashland, USA**.** Hydroprpylmethylcellulsoe 5 cps was from Dow Chemicals Co., USA**.** Ethanol was from Merck, Germany**,** Methanol was from Sabic, Saudi Arabia**,** methylene chloride was from Ineor Chlor Ltd, UK**,** acetone was from Merck, Germany and butylated hydroxy toluene (BHT) was from Scarlab S.L, Spain.

Drug-excipients compatibility study. The physical and binary mixture of actives and carriers were fixed at a ratio of 1:1.[13] The compatibility study was carried out further through Fourier Transform Infrared Spectroscopy (FT-IR) and Differential Scanning Calorimetric (DSC) analyses.

Preparation of solid dispersions. Solid dispersions of LDV were prepared with poloxamer 188, poloxamer 407, Klucel™ EF, Klucel™ EXF, HPMC 5cps, povidone K17, povidone K30 and carboxymethylcellulose (CMC) sodium at a drug: polymer ratio of 1:1. Respective amount of carrier (except CMC sodium and HPMC) was dissolved in a glass beaker containing ethanol and the drug was added in parts with continuous stirring (Table 1). Because of the insolubility of HPMC and CMC sodium in ethanol, a 50:50 ratio of ethanol: dichloromethane was used as the solvent. In all cases, the concentration of the solution was 10% w/w in the solvent. Then the solvent was removed by evaporation at 40°C under a vacuum using Eppendorf Vacuum Concentrator. The solid dispersion (SD) preparations were crushed with a mortar and pestle. Then the crushed preparations were sieved through a 0.60 mm (#30 mesh) screen to get uniform particles for convenience in the next processing steps. The SD preparations were then dried at 60°C in a vacuum oven overnight to achieve the desired level of residual solvent. The prepared SDs were characterized by DSC to check for their crystallinity and amorphousity. The formulations are presented in the table 1.

Preparation of nanosuspensions. From the DSC curves of all solid dispersion formulations, it could be understood that ledipasvir transformed from the crystalline to amorphous form in solid dispersions manufactured with poloxamer 188 (F1SD), poloxamer 407 (F2SD), Klucel™ EF (F3SD), Klucel™ EXF (F4SD) and HPMC 5cps (F5SD). Ledipasvir remained in the crystalline form in the solid dispersions prepared with povidone K17

(F6SD), povidone K 30 (F7SD), and CMC Na (F8SD).

Table 1. The formulation for solid dispersions of ledipasvir.

Formula	API	Polymer	Ratio
F1SD	Ledipasvir	Poloxamer 188	1:1
F2SD		Poloxamer 407	
F3SD		HPC (Klucel EF)	
F4SD		HPC (Klucel EXF)	
F5SD		HPMC 5cps	
F6SD		PVP K 17	
F7SD		PVP K 30	
F8SD		CMC Na	

Hence, for the preparation of nanosuspension, poloxamer 188 and poloxamer 407 were selected. From Klucel™ EF and EXF, Klucel™ EXF was selected as it is finer in size. HPMC 5cps was selected as it has lower molecular weight and viscosity which make it more suitable for immediate-release formulations.[14]

The experimental design for the formulation was fabricated using the D-optimal design (Table 2). The design and the results were statistically evaluated using Design Expert® software (version 13). The proposed formulations were developed using mixtures of API and polymer. The API and the polymer in the mixture were selected as categorical factors whereas the particle size and polydispersity index (PDI) were selected as the response factors. Therefore, as a first step of the preparation of nanosuspensions, solid dispersions were prepared in three drug: polymer ratios for each of the polymers (Table 3).

Table 2. Ratios of API and polymers for the D-optimal mixture design.

Parameters	API	Polymer	Total
Actually used (g)	0.7	1.3	2
Interpretation for D-optimal (%)	35	65	100
Actually used (g)	1	1	2
Interpretation for D-optimal (%)	50	50	100
Actually used (g)	1.3	0.7	2
Interpretation for D-optimal (%)	65	35	100

Table 3. Solid dispersion (SD) formulation with different ratios of Ledipasvir and polymers.

API	Polymers	Ratio	SD suspension in water
Ledipasvir	Poloxamer 188	0.7:1.3	NSF1a
		1:1	NSF1b
		1.3:0.7	NSF1c
	Poloxamer 407	0.7:1.3	NSF2a
		1:1	NSF2b
		1.3:0.7	NSF2c
	HPC (Klucel™ EXF)	0.7:1.3	NSF4a
		1:1	NSF4b
		1.3:0.7	NSF4c
	HPMC 5cps	0.7:1.3	NSF5a
		1:1	NSF5b
		1.3:0.7	NSF5c

All the solid dispersion preparations were distributed in water separately at a concentration of 1g / 5 ml. Then ultra-sonicated for 5, 10 and 15 minutes with occasional stirring to find out the optimum time to prepare lump-free smooth dispersion.

Characterization of nanosuspensions.

Measurement of viscosity. To measure the viscosity of the formulations, 0.5 ml of each suspension was taken in a Brookfield viscometer using spindle no. 40 and 0.1 rpm. As the current suspension is formulated considering also the pediatric and geriatric population, a viscosity ranging from 1090 cps to 1240 cps can be considered optimum in terms of dosing accuracy to avoid side effects of overdose or low efficacy of underdose based on the study of some pediatric market preparations.[15]

Determination of zeta potential. The Zeta potential of each of the SD suspensions was determined through a Zetasizer ZS90. Samples were suitably diluted using water and redispersed by gentle shaking. Then the zeta potential of the samples was measured after 10 runs in triplicates using a clear disposable zeta cell.[16]

Particle size distribution (PSD) and polydispersity index (PDI) determination. PSD and PDI were obtained with the help of a Zetasizer ZS90.

Samples were suitably diluted using water and redispersed by gentle shaking. The mean particle size diameter and PDI of the samples were measured after 25 runs using a disposable sizing cuvette in triplicate.

Physical stability of the nanosuspension. The physical stability of all the nanosuspensions of ledipasvir was checked. All the suspensions were kept undisturbed in test tubes at room temperature. Visual observations were made after 1, 2, 3 and 4 weeks along with the redispersibility of the suspensions.

Stabilization of nanosuspensions. One of the major stability problems of the nanosuspension is the aggregation of particles resulting in hard cake formation. By gelling the continuous phase, with the help of appropriate excipients, this type of suspension can be made kinetically stable.[17] Examples of such agents are alginates, HPMC, xanthan gum, CMC Sodium etc. The increase in stability can be confirmed by measuring the zeta potential.[18,19] However, zeta potential can also be modified by adding citric acid, sodium citrate in the preparations to increase the suspension stability.[20]

Design of experiment for stabilization of prepared nanosuspensions. The response surface methodology (RSM) is applied for designing the experiment of stabilization of prepared nanosuspension. As Box-Behnken design (BBD) is most effective in the case of such designs, a 3 factors and 3 levels BBD was applied in the current study.[21]

Dissolution method. Ledipasvir is an INN molecule and thus the analytical method for ledipasvir is not available in any official pharmacopoeia. An in-house UV-Vis spectrophotometric method was developed to analyse the extent of dissolution of ledipasvir from its finished pharmaceutical product. The method was validated following ICH Q2 (R1) guidelines. The sample solution was scanned over a range of 250–400 nm in triplicates to find out the maximum absorbance. The USP type II apparatus with 75 rpm and an appropriate dissolution medium of pH 6.0 were utilized during dissolution.[22]

***In vivo* simulation study.** The *in-vivo* simulation was made with the help of a convolution process to obtain the blood concentration of ledipasvir from the dissolution profiles of the formulated best suspensions and market product. The aim was to simulate and compare the pharmacokinetic parameters (peak plasma concentration, C_{max} and area under the curve, $AUC_{(0\ to\ t)}$).

C_{max} and $AUC_{(0\ to\ t)}$ were predicted using PKSolver® which works on the principle of convolution and deconvolution.[23] At first, the predicted values of C_{max} and AUC were obtained for the market product (using one tablet of a dose of 90 mg of ledipasvir). These predicted values were compared with the experimental C_{max} and AUC obtained from the studied literature. Then, the predictability of the model was evaluated. The following equation was applied to calculate the percent prediction error (% PE) for C_{max} and AUC:

$$\%PE = [(\text{Observed value} - \text{Predicted value}) / \text{Observed value}] \times 100$$

A %PE value ≤ 10% ensures the predictability of the model. A %PE value between 10% and 20% indicates inconclusive predictability and needs further data. A %PE value >20% indicates insufficient or lack of predictability.[24,25]

Stability study of the formulated suspensions. Physical and chemical stabilities of the final formulations of ledipasvir, FNSF1a and FNsF2a were carried out at 40°C ± 2°C and 75% ± 5% RH after three and six months.[26,27] For physical stability, zeta potential values, sedimentation volume and redispersibility were assessed.[28] For chemical stability, assay and dissolution profiles were checked. After six months of stability studies, assay results were compared with the initial assay results by one-way analysis of variance (ANOVA) using Minitab®, version 17.[29,30] ANOVA is used to compare the stability results of the drug product.[29,31] DDSolver® software was used to compare the dissolution results for similarity factor (f_2), difference factor (f_1) and dissolution efficiency (% DE).

RESULTS AND DISCUSSION

Drug-excipients compatibility study. The prominent peaks of ledipasvir were observed in the region 1660 cm^{-1} due to >N-H (secondary amine NH bend), 1288 cm^{-1} due to –C-N (primary amine, CN stretch), 1240 cm^{-1} due to –C-C (vibration), 1098 cm^{-1} due to –C-N (primary amine, CN stretch), 1040 cm^{-1} due to cyclohexane ring vibrations.

All the principal peaks of drug and excipients are visible in the FT-IR spectrum of ledipasvir, excipients, and physical mixture of drug & excipients. No significant shifting of the peaks of the mixtures was observed compared to their data and thus confirms good compatibility between drug and polymers.

From the DSC results of ledipasvir, a sharp endothermic peak was found at 172.91°C due to the melting of the API. From the DSC curves of ledipasvir and all the LDV-polymer binary mixtures, it could be understood that there was no interaction between the API and the excipients.

Characterization of prepared solid dispersions. The prepared solid dispersions (SDs) were characterized by DSC to check for their crystallinity and amorphousity (Figure 1). From the DSC curves of all solid dispersion formulations, it could be understood that ledipasvir has been converted from the crystalline to the amorphous form in solid dispersions prepared with poloxamer 188 (F1SD), poloxamer 407 (F2SD), Klucel™ EF (F3SD), Klucel™ EXF (F4SD) and HPMC 5cps (F5SD), as the characteristic peak of ledipasvir is absent in the solid dispersion. Ledipasvir remained in the crystalline form in the solid dispersions prepared with povidone K 17 (F6SD), povidone K 30 (F7SD), and CMC Na (F8SD).

Characterization of prepared nanosuspensions.

Particle size distribution (PSD). From the obtained results it can be said that solid dispersion suspensions NSF1(a), NSF1(b) and NSF1(c), NSF2(a), NSF2(b) and NSF2(c), NSF4(a), NSF4(b) and NSF4(c) are nanosuspensions, as their particle size ranges within 1000 nm (Figure 2). However, a further investigation of NSF4(a), NSF4(b) and NSF4(c) revealed that these formulations contain a significant number of particles outside the nanosuspension range which makes them unacceptable. Moreover, NSF5(a), NSF5(b) and NSF5(c) did not produce nanosuspension, as all of them have particle sizes over 1000 nm.

Polydispersity index (PDI). From the results, it can be said that SD suspensions, NSF1(a), NSF1(b), NSF1(c), NSF2(a), NSF2(b), NSF2(c), NSF4(a), NSF4(b) and NSF4(c) are nanosuspensions in terms of the definition of nanosuspension (Figure 3). Nevertheless, NSF4(a), NSF4(b) and NSF4(c) have PDI over 0.7 which indicates a very wide range of particle size distribution and are not suitable to be measured by a Zetasizer (photon correlation spectroscopy). From the results of PSD and PDI, it was also found that nanosuspensions, NSF1(a) and NSF2(a) produced the best results as they had the lowest particle size with acceptable PDI values.

Viscosity. From the obtained values, it can be said that the viscosity of the suspensions decreased gradually with a gradual decrease of polymer concentrations for HPC (Klucel™ EXF) and HPMC 5cps (Figure 4). In the case of poloxamers, viscosity was maximum at 50% polymer concentration whilst the viscosity was lower for both 65% and 35% polymer concentrations.

Most of the suspensions had a viscosity lower than 200 cps which is not ideal for an oral suspension. Only NSF5a, 5b and 5c had viscosity above 200 cps. Although the suspensions were easily pourable, the lower viscosity may cause too fast sedimentation, leading to the problem of dose uniformity. Furthermore, there are chances of hard cake formation upon storage for a longer period which may cause dose uniformity and bioavailability problems.

Zeta potential. The zeta potential of all the SD suspensions was measured using a Zetasizer (Figure 5). Furthermore, a deep insight was developed by analyzing the zeta potential of each formulation (Figure 6). From the data, it can be viewed that all the SD suspensions had very low zeta potentials. It

indicates that all the suspensions were unstable. There were possibilities of rapid settling, agglomeration and hard cake formation upon storage. Hence, the formulations needed to be stabilized using some appropriate techniques.

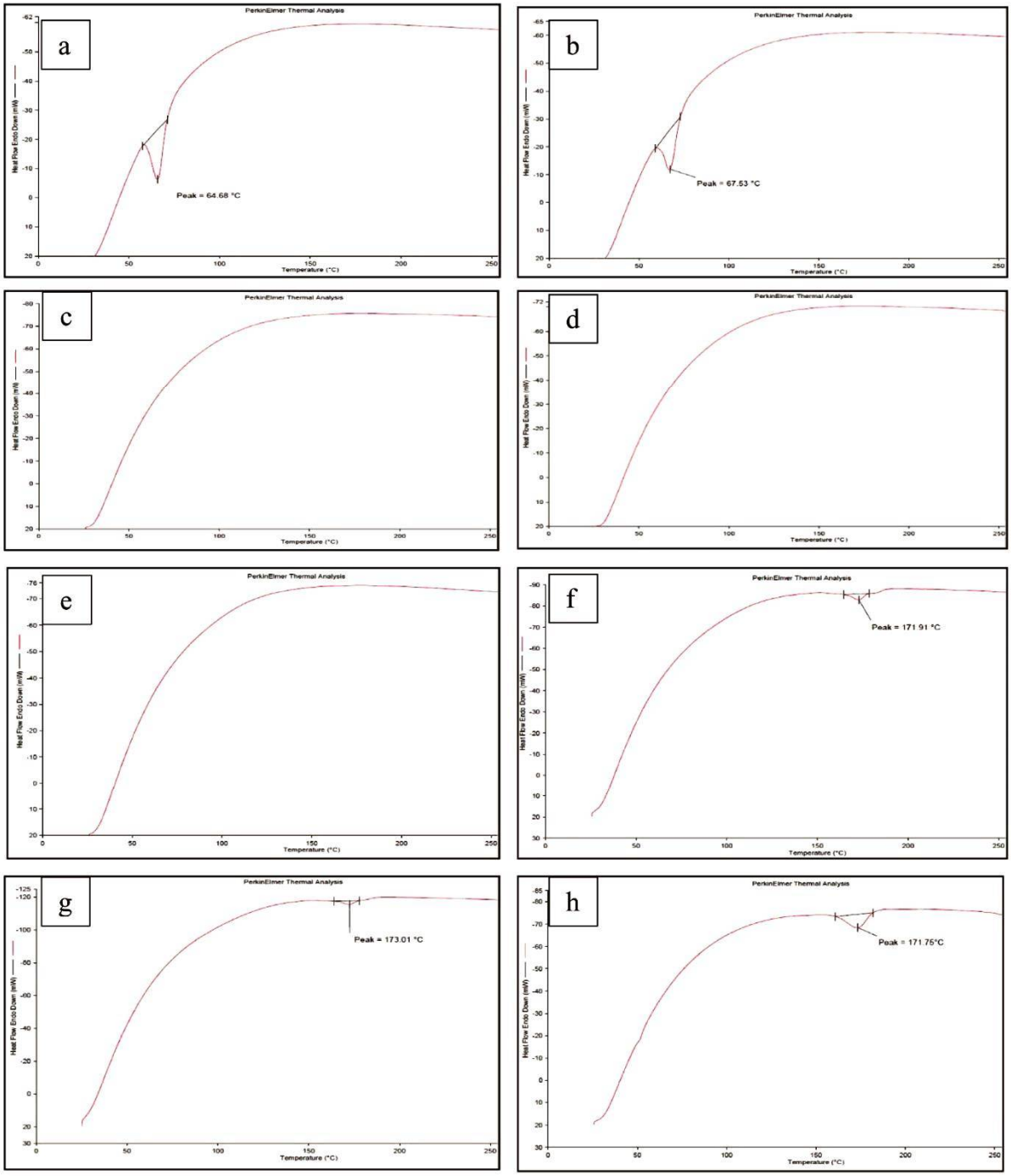

Figure 1. DSC curve of a) Ledipasvir-Poloxamer 188 solid dispersion (F1 SD); b) Ledipasvir-Poloxamer 407 solid dispersion (F2 SD); c) Ledipasvir-Klucel™ EF solid dispersion (F3 SD); d) Ledipasvir-Klucel™ EXF solid dispersion (F4 SD); e) Ledipasvir-HPMC 5cps solid dispersion (F5 SD); f) Ledipasvir-PVP K17 solid dispersion (F6 SD); g) Ledipasvir-PVP K30 solid dispersion (F7 SD); h) Ledipasvir-CMC Sodium solid dispersion (F8 SD).

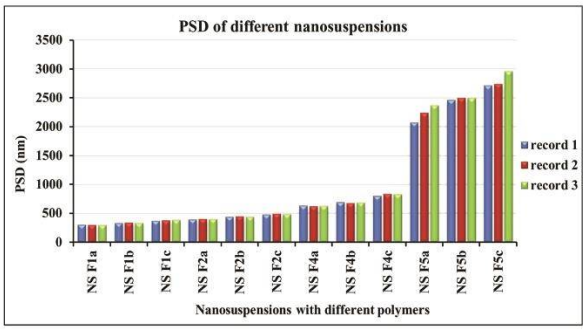

Figure 2. Particle size distribution of formulated different nanosuspensions.

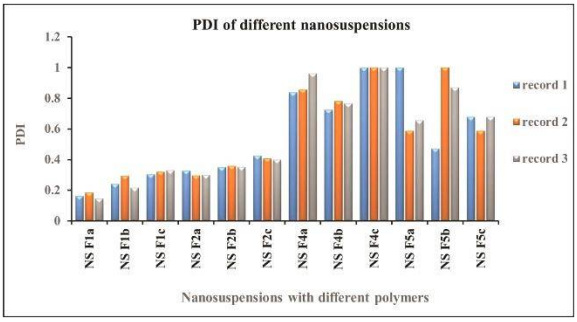

Figure 3. PDI of different nanosuspensions.

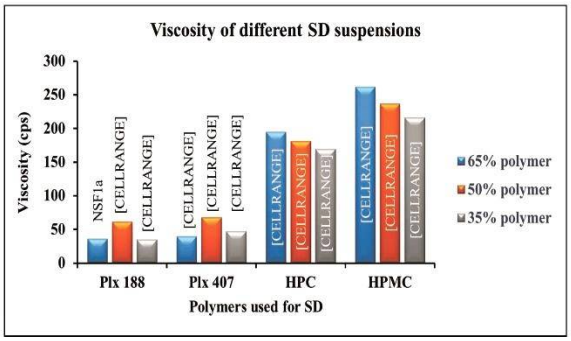

Figure 4. The viscosity of all SD suspensions.

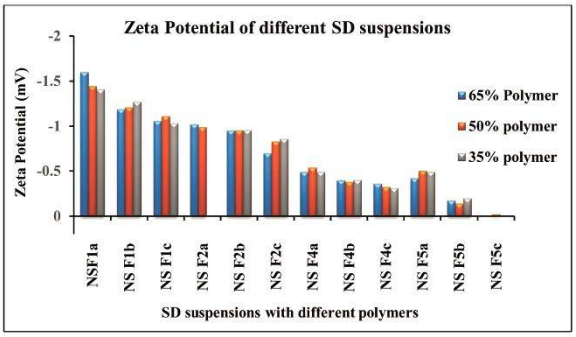

Figure 5. Zeta potential of different SD suspensions.

Visual observation for ledipasvir SD suspensions. From the observation it was found that after 4 weeks of storage sediment volume remained constant and hard cake formed. The suspension was not re-dispersible upon shaking. So, none of the prepared SD suspensions were physically stable upon storage.

QbD for preparation of ledipasvir nanosuspension.

Results for formulation NSF1. Three experimental runs were designed and experimented with based on different compositions. Results obtained for responses of the study response 1 (PSD) and response 2 (PDI) are mentioned (Table 4).

Table 4. Factors and responses for NSF1 ledipasvir nanosuspension.

Run	Factor A (Ledipasvir, %)	Factor B (Poloxamer 188, %)	Response 1 (PSD) nm	Response 2 (PDI)
1	35	65	299.1	0.164
2	50	50	335.1	0.251
3	65	35	379.7	0.319

Analysis of responses for NSF1. Statistical analysis revealed that both responses 1 and 2 followed the linear model. The model F-value of response 1 (263.51) and response 2 (199.65) implies the significance of both models. There are only a 3.92% and 4.5% chances that an F-value this large could occur due to noise.

Optimization of formulation NSF1: To optimize the responses constraints were set (Table 5). Maximum desirability was the criteria for the optimization of the formulation. Formulation containing 35% of the drug and 65% of poloxamer 188 was selected as the optimized formulation for NSF1 with a predicted PSD of 297.667 and PDI of 0.167.

Table 5. Constraints for optimization of NSF1.

Name	Goal	Lower limit	Upper limit
A:Ledipasvir	Within range	35	65
B:Poloxamer 188	Within range	35	65
PSD	Minimize	299.1	379.7
PDI	Minimize	0.164	0.319

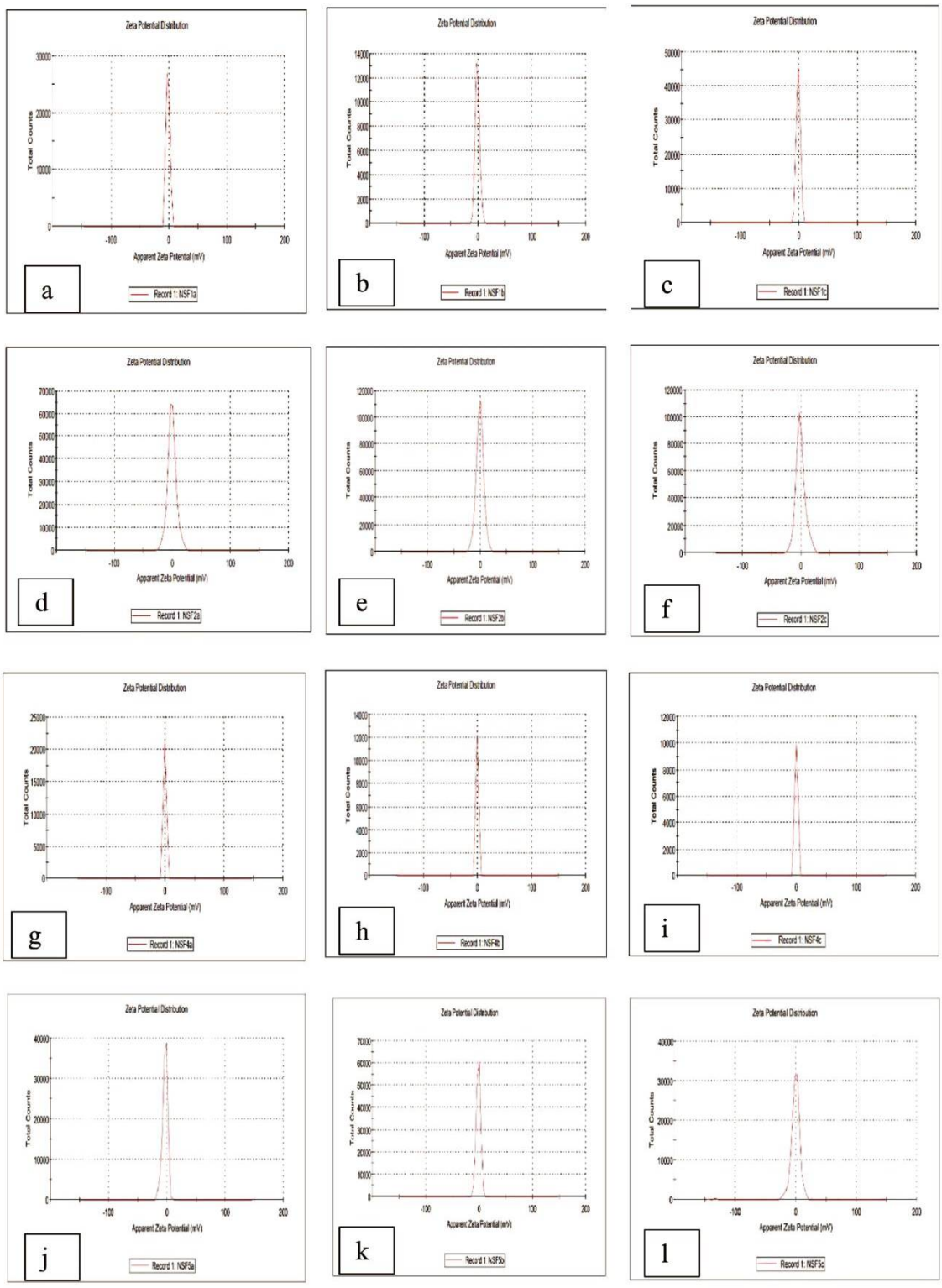

Figure 6. Zeta potential distribution of (a) NSF1a SD suspension; (b) NSF1b SD suspension; (c) NSF1c SD suspension; (d) NSF2a SD suspension; (e) NSF2b SD suspension; (f) NSF2c SD suspension; (g) NSF4a SD suspension; (h) NSF4b SD suspension; (i) NSF4c SD suspension; (j) NSF5a SD suspension; (k) NSF5b SD suspension; (l) NSF5c SD suspension.

Results for formulation NSF2. Three experimental runs were designed and experimented with based on different compositions. Results obtained for response 1 (PSD) and response 2 (PDI) are mentioned (Table 6).

Table 6. Factors and responses for NSF2 ledipasvir nanosuspension.

Run	Factor A (Ledipasvir, %)	Factor B (Poloxamer 407, %)	Response 1 (PSD) nm	Response 2 (PDI)
1	35	65	400	0.309
2	50	50	442.9	0.355
3	65	35	486.8	0.411

Analysis of responses for NSF2. Statistical analysis revealed that both response 1 and response 2 as for NSF1 followed a linear model. The model F-value of response 1 (22602.72) and response 2 (312.12) implies that both models are significant. There are only 0.42% and 3.6% chances that an F-value this large could occur due to noise.

Optimization of formulation NSF2. To optimize the responses for NSF2, at first constraints were set (Table 7). The formulation optimization was done based on maximum desirability. Formulation containing 35% of the drug and 65% of poloxamer 407 was selected as the optimized formulation for NSF1 with a predicted PSD of 399.833 and PDI of 0.307.

Table 7. Constraints for optimization of NSF2.

Name	Goal	Lower limit	Upper limit
A:Ledipasvir	Within range	35	65
B:Poloxamer 407	Within range	35	65
PSD	Minimize	400	486.8
PDI	Minimize	0.309	0.411

Results for formulation NSF4. Three experimental runs were designed and experimented with based on different compositions. Results obtained for responses of the study response 1 (PSD) and response 2 (PDI) are mentioned in table 8. The model F-value of 16.36 implies the model is not significant relative to the noise. There is a 15.43% chance that an F-value this large could occur due to noise.

Table 8. Factors and responses for NSF4 ledipasvir nanosuspension.

Run	Factor A (Ledipasvir, %)	Factor B (HPC, %)	Response 1 (PSD) nm	Response 2 (PDI)
1	35	65	629.8	0.886
2	50	50	685.6	0.758
3	65	35	825	1

Results for formulation NSF5. Three experimental runs were designed and experimented with based on different compositions. Results obtained for responses of the study response 1 (PSD) and response 2 (PDI) are mentioned in table 9. The model F-value of 80.88 implies that the model is not significant relative to the noise. There is a 7.05% chance that an F-value this large could occur due to noise.

Table 9. Factors and responses for NSF5 ledipasvir nanosuspension.

Run	Factor A (Ledipasvir, %)	Factor B (HPMC 5cps, %)	Response 1 (PSD) nm	Response 2 (PDI)
1	35	65	2268	0.748
2	50	50	2483.7	0.781
3	65	35	2802.3	0.649

Responses analyzed using ANOVA, were found significant for NSF1 and NSF2. Models suggested that the responses for NSF4 and NSF5 were insignificant. Therefore, these two formulations were then removed from further study, and the optimization of NSF1 and NSF2 were done to have the best responses. Afterwards, NSF1a and NSF2a were found as optimized batches of the experiments based on the desirability of the models which were then taken for further studies. It is to be noted that the formulation having a higher concentration of the polymer exhibited the most desirable results as they had the lowest PSD and PDI.

Stabilization of prepared nanosuspension. For stabilizing the nanosuspension, a suspension vehicle was incorporated following the Box-Behnken experimental design (Tables 10 and 11).

Table 10. Factors for optimization of suspension vehicle.

Factors	Level		
	-1	0	+1
Xanthan gum (g)	0.1	0.3	0.5
Avicel® RC-591 (g)	0.25	0.5	0.75
Citric acid monohydrate (g)	0.3	0.6	0.9

Analysis of response 1 (viscosity, cp). Response 1 followed a linear model with an adjusted R^2 value of 0.8739 and a predicted R^2 value of 0.7937. The amount of xanthan gum (factor A) and avicel RC 91 (factor B) played a significant role on response 1 (Figures 7a and 7b).

The model F-value of 28.71 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

Analysis of response 2 (zeta potential, mv). Response 2 followed a quadratic model with an adjusted R^2 value of 0.9955. Amount of xanthan gum (factor A), avicel RC 91 (factor B), citric acid (factor C) and squared term of factor C played a significant role in response 2 (Figure 7c and 7d).

Table 11. Experimental runs using Box-behnken design and the observed responses for stabilization of prepared nanosuspensions.

Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2
	A:Xanthan gum (g)	B:Avicel RC 591 (g)	C:Citric acid (g)	Viscosity (cp)	Zeta potential (mv)
1	0.500	0.250	0.6	926.0	-30.0
2	0.100	0.250	0.6	792.0	-29.0
3	0.500	0.750	0.6	1226.0	-32.0
4	0.300	0.500	0.6	1016.0	-30.0
5	0.100	0.500	0.9	856.0	-18.0
6	0.300	0.250	0.3	934.0	-36.0
7	0.300	0.750	0.3	1097.0	-37.0
8	0.300	0.750	0.9	1088.0	-19.0
9	0.100	0.500	0.3	839.0	-36.0
10	0.100	0.750	0.6	907.0	-31.0
11	0.500	0.500	0.3	1124.0	-37.0
12	0.300	0.250	0.9	965.0	-18.0
13	0.500	0.500	0.9	1141.0	-20.0

The model F-value of 294.23 implies the model is significant. There is only a 0.03% chance that an F-value this large could occur due to noise.

Optimization of suspension vehicle. To optimize the suspension vehicle, constraints were set at first (Table 12). Among 54 solutions found from the software, the one with maximum desirability of 0.973 was selected as the optimized solution consisting of xanthan gum 0.5 g, avicel RC 591 0.750 g and citric acid 0.366 g. The predicted viscosity and zeta potentials were 1203.201 cps and -37 mv, respectively.

Table 12. Constraints for optimization of suspension vehicle.

Name	Goal	Lower limit	Upper limit
A:Xanthan gum	Within range	0.1	0.5
B:Avicel RC 591	Within range	0.25	0.75
C:Citric acid	Within range	0.3	0.9
Viscosity	Maximize	792	1226
Zeta potential	Minimize	-37	-18

Preparation of stabilized suspensions of ledipasvir. NSF1a and NSF2a nanosuspensions were selected for the stabilization experiment as they produced the best results in terms of PSD and PDI.

NSF1a and NSF2a nanosuspensions of ledipasvir were added with the suspension vehicle in the ratio of 25.71 ml of nanosuspension and 74.29 ml of suspension vehicle so that each 5 ml suspension contains 90 mg of ledipasvir.

Evaluation of stabilized suspension.

Determination of zeta potential. When the zeta potential value of the suspension vehicle becomes more than -30, it indicates the physical stability of the vehicle. The zeta potential values of all formulated suspensions of ledipasvir were more than or equal to -30 which denoted that the suspension had become stable after adding to the suspension vehicle (Table 13).

Table 13. Zeta potentials of the suspension vehicle and stabilized nanosuspensions.

Formulation	Zeta potential (mv)			
	Run 1	Run 2	Run 3	Average
Suspension vehicle	-36.6	-36.7	-36.4	-36.6
FNSF1a	-34.3	-34.2	-33.8	-34.1
FNSF2a	-33.9	-34.1	-33.5	-33.8

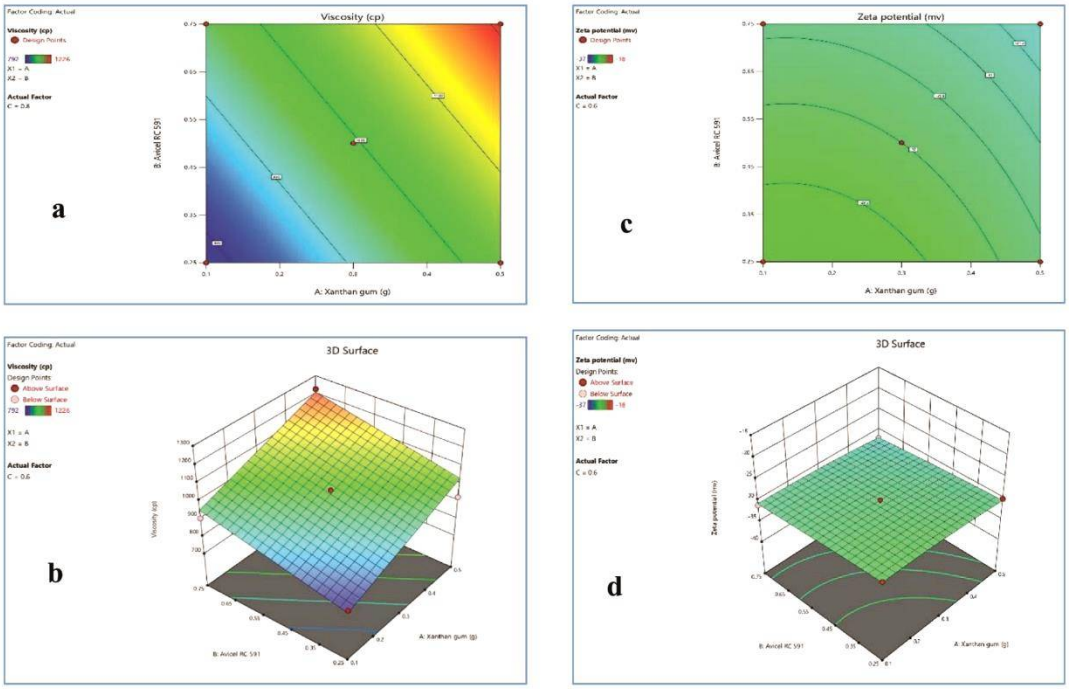

Figure 7. (a) Contour plot and (b) 3D response surface plot for response 1 (viscosity) for optimization of suspension vehicle; (c) Contour plot and (d) 3D response surface plot for response 2 (zeta potential) for optimization of suspension vehicle.

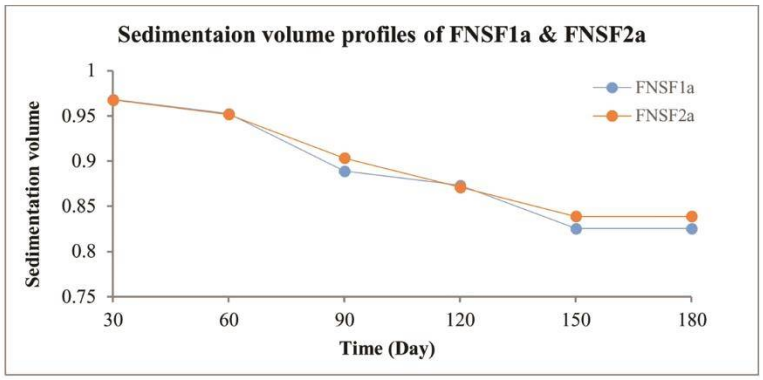

Figure 8. Sedimentation volume profiles of FNSF1a and FNSF2a suspensions.

Sedimentation volume of stabilized suspension. The sedimentation volumes (F) of the formulated ledipasvir suspensions FNSF1a and FNSF2a at different time points (day 30, 60, 90, 120, 150 and 180) are presented in figure 8.

From the sedimentation volumes, it was found that formulated suspensions of ledipasvir FNSF1a and FNSF2a have F values of 0.83 and 0.84, respectively. This indicates that the prepared suspensions are stable and flocculated with fewer chances of cake formation, and should be easily redispersible. These will ensure dose uniformity while using by the patients.

Dissolution profiles of the stabilized suspensions. Dissolution profiles of the stabilized suspensions FNSF1a and FNSF2a were assessed (Tables 14 and 15). The label claim for both cases were 'Each 5 ml suspension contains 90 mg of ledipasvir'. From the observed data it can be said that formulated nanosuspensions FNSF1a and FNSF2a had significantly faster dissolution rates than the market product 2 (Figure 9 and table 14).

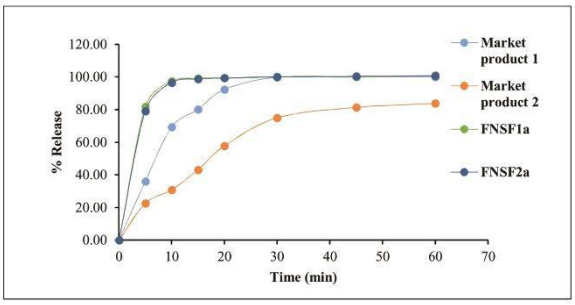

Figure 9. Dissolution profiles of different preparations of Ledipasvir.

Table 14. Dissolution profile comparison with similarity factor (f_2), difference factor (f_1) and dissolution efficiency (% DE).

Products	Difference factor (f_1)	Similarity factor (f_2)	Dissolution efficiency (% DE)
Market product 1	-	-	48.46
Market product 2	31.76	29.13	24.98
FNSF1a	17.59	34.58	76.30
FNSF2a	16.79	35.65	75.01

Table 15. R^2 values of different mathematical models obtained for studied preparations.

Mathematical model	MP1	MP2	FNSF1a	FNSF2a
Zero order plot	0.5887	0.8406	0.2860	0.3003
First order plot	0.6630	0.9444	0.4848	0.2885
Korsmeyer-Peppas plot	0.0619	0.0155	0.1623	0.1579
Higuchi plot	0.8451	0.9619	0.5756	0.5930
Hixson plot	0.8760	0.9165	0.6699	0.7825

***In vivo* simulation study.** Predicted pharmacokinetic parameters were determined for MP1, FNSF1a and FNSF2a, and a paired t-test was done for comparison. The results are listed in Tables 16 and 17.

From table 16, it can be said that the *p*-values for the t-test of both FNSF1a and FNSF2a with the market product 1 (MP1) are greater than 0.05 for both C_{max} and AUC. Therefore, it can be concluded that both formulated suspensions, FNSF1a and FNSF2a, have similar *in-vivo* performance compared to the market product (MP1) for a dose of 90 mg. However, this prediction is based on a simulation study and more insights are needed from an actual *in-vivo* study.

From table 17, it was found that the p-values for the t-test of both FNSF1a and FNSF2a with the MP1 are smaller than 0.05 for both C_{max} and AUC. Therefore, it can be concluded that both formulated suspensions, FNSF1a and FNSF2a, have significantly different *in-vivo* performance compared to the market product (MP1). The absorption of ledipasvir increased to 1.65 fold for FNSF1a and to 1.31 fold for FNSF2a compared to the market product MP1 at a dose of 270 mg.

Afterwards, the predicted pharmacokinetics profiles were compared for 3 tablets of the market product 1, and 15 ml of each FNSF1a and FNSF2a suspensions (Table 17)**.**

Stability study.

Zeta potential. Zeta potentials of the formulated suspensions of ledipasvir, FNSF1a and FNSF2a were checked after 3 and 6 months at 40°C ± 2°C and 75%

± 5% RH. The zeta potentials of FNSF1a and FNSF2a were more than or equal to -30, indicating the physical stability of the formulated suspensions.

Assay content. Assay content of ledipasvir in the formulated suspensions after 3 and 6 months found that there were no significant changes in the formulated suspensions of ledipasvir (FNSF1a and FNSF2a) in terms of assay content compared to the initial results. Percentages of relative standard deviation were found within the limits. One-way ANOVA was done using Minitab® version 17 to compare the assay results for further evaluation (Table 18). From the ANOVA, it was found that the *p*-values were 0.088 for FNSF1a and 0.398 for FNSF2a. The obtained *p*-values were greater than 0.05, indicating that there were no significant changes as per ICH Q1(R2) guideline in the formulated suspensions in terms of assay of ledipasvir after six months of the accelerated study compared to the initial results presented**.** Thus, the formulations appeared to be stable over the entire shelf-life period.

Table 16. Predicted PK parameters for FNSF1a and FNSF2a suspensions for 90 mg dose.

PK parameters	Predicted values			p-value for paired t-test	
	MP1	FNSF1a	FNSF2a	FNSF1a	FNSF2a
C_{max} (ng/ml)	300.37 ± 4.19	300.26 ± 3.12	299.75 ± 4.81	0.875	0.225
$AUC_{(0\ to\ t)}$ (ng.h/ml)	10336.10 ± 144.19	10438.05 ± 51.87	10404.83 ± 67.01	0.196	0.263

Table 17. Predicted PK parameters for MP1, FNSF1a and FNSF2a for a dose of 270 mg.

PK parameters	Predicted values			p-value for the paired t-test	
	MP1	FNSF1a	FNSF2a	FNSF1a	FNSF2a
C_{max} (ng/ml)	177.38 ± 2.89	293.23 ± 1.77	233.13 ± 2.18	0.000	0.000
$AUC_{(0\ to\ t)}$ (ng.h/ml)	6343.39 ± 27.10	10399.61 ± 34.53	8885.97 ± 32.94	0.000	0.000

Table 18. Assay content of the formulated suspensions of ledipasvir FNSF1a and FNSF2a after 3 and 6 months at accelerated conditions.

Samples	FNSF1a			FNSF2a		
	Initial	3 months	6 months	Initial	3 months	6 months
Sample 1	99.38	100.70	98.62	98.49	98.51	99.29
Sample 2	98.92	100.13	98.38	99.77	100.07	98.58
Sample 3	99.49	100.44	100.29	99.11	100.23	98.36
Average	99.26	100.42	99.10	99.12	99.60	98.74
Standard deviation	0.30	0.29	1.04	0.64	0.95	0.49
% RSD	0.30	0.28	1.05	0.65	0.95	0.49
p-value		0.088			0.398	

Redispersibility. Both FNSF1a and FNSF2a were easily re-dispersible upon shaking and there was no hard cake formation after 3 and 6 months of accelerated stability study.

Dissolution profiles. Dissolution profiles of the formulated suspensions were checked after 3 and 6 months keeping the formulations at accelerated conditions (40℃ ± 2℃ and 75% ± 5% RH) to confirm the consistency with initial preparations. The release of the drug from the suspensions were still the same even after six months of exposure to the accelerated stability conditions which indicates that the formulations were stable throughout the period of storage.

Evaluation of similarity (f2) and difference (f1) factor after stability study. The dissolution

results of preparations FNSF1a and FNSF2a after 3 and 6 months of stability period were then compared with the initial dissolution results for similarity factor (f2), difference factor (f1) and dissolution efficiency (%DE). From the data, it was found that for both FNSF1a and FNSF2a, similarity factors were well above 50 and the different factors were well below 15 at both 3 and 6 months time points (Table 19). Moreover, the %DE for both FNSF1a and FNSF2a were well within ± 10% at both 3 and 6 months time points compared to their respective initial dissolution results. Therefore, the dissolution profiles after the stability study period can be considered similar to that of the initial.

Table 19. Dissolution profile comparison with similarity factor (f2), difference factor (f1) and dissolution efficiency (% DE).

Time points	FNSF1a			FNSF2a		
	f1	f2	% DE	f1	f2	% DE
Initial	-	-	76.31	-	-	75.02
3 months	1.69	83.34	74.88	1.64	86.53	73.58
6 months	2.60	78.34	73.92	2.83	75.30	72.47

CONCLUSION

As ledipasvir shows high absorption but low dissolution rates, dissolution is the rate-limiting step for oral absorption of this drug. Currently, two dosage forms of ledipasvir, tablet and pellets, are available in the market. The tablets can be taken with or without food. Pellets are intended for children who face difficulty swallowing the tablet formulation. But the administration of the pellets is also complicated, as it is indicated to sprinkle the pellets on one or more spoonsful of non-acidic soft food at or below room temperature. Therefore, this process is complicated for those who are unable to swallow tablets. To eradicate this complexity, solid dispersion-based nanosuspensions were prepared and finally stabilized as oral liquid suspensions which can be conveniently administered by geriatric and pediatric populations or patients having difficulty with swallowing. Poloxamer 188, poloxamer 407, HPC (Klucel™ EXF) and HPMC 5 cps were selected for the preparation of nanosuspensions, as ledipasvir was converted to an amorphous state from the crystalline form in the SDs prepared with these polymers. Then the prepared nanosuspensions were evaluated for viscosity, zeta potential, particle size distribution (PSD), polydispersity index (PDI) and redispersibility. From the PSD and PDI studies, it was found that preparations with different ratios of poloxamer 188 and poloxamer 407 produced acceptable results. From the viscosity, zeta potential and redispersibility studies, it was found that all the prepared nanosuspensions were physically unstable due to very low viscosity, zeta potential and hardening of sediment resulting in the inability to redisperse. However, the best results were found with poloxamer 188 (NSF1a) and poloxamer 407 (NFS2a) at a ratio of 0.7:1.3 for API: polymer in terms of PSD and PDI. These nanosuspensions (NSF1a and NFS2a) were then stabilized by increasing viscosity, incorporating a gel network and altering the surface activity i.e. zeta potential through the inclusion of a suspension vehicle. The suspension vehicle was developed using the Box-Behnken design. Dissolution study showed that formulated suspensions, FNSF1a and FNSF2a had a significantly faster dissolution rate than market product 2 (MP2). The sedimentation volume of the formulated suspensions, FNSF1a and FNSF2a were assessed and it was found that they were highly flocculated suspensions with excellent redispersibility for up to six months. At the same time, *in-vivo* simulation was done with the help of PKSolver® and it was found that the absorption of ledipasvir was similar to that of the market product 1 (MP1) for a dose of 90 mg, whereas its absorption increased to 1.65 fold for formulated suspensions FNSF1a and to 1.31 fold for FNSF2a compared to the market product MP1 at a dose of 270 mg. Finally, a stability study was conducted for both suspensions FNSF1a and FNSF2a at accelerated conditions for up to six months and samples were assessed at 3 months and 6 months time points to check for zeta potential, redispersibility, assay and dissolution. Interestingly, both formulations were to be found stable throughout the study period.

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