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Formulation and Evaluation of Favipiravir Proliposomal Powder for Pulmonary Delivery by Nebulization

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ABSTRACT

The favipiravir proliposomal is a powder formulation for pulmonary delivery by nebulization, prepared and characterized by film deposition on carrier method. The formulations have good flow properties and upon hydration, liposome vesicles were seen within 2-3 minutes. According to the results of SEM studies the surface morphology was coarse and non-porous. The DSC and XRD studies confirm that the favipiravir drug is crystalline, whereas the FPL6 is amorphous. In-vitro nebulization studies performed using a twin stage impinger (TSI) revealed that the nebulization time ranges from 12.95 to 14.3 minutes. The aerosol mass output and rate ranges from 44.31 to 54.28 %, and 0.17 to 0.22 mg/min. respectively. The respirable fraction ranges from 8.14 to 12.31%. The FPL6 formulation has a higher respirable fraction of 12.31%, which is available for therapeutic action in the lungs and stable. The results showed that the favipiravir proliposomal formulation was suitable for pulmonary drug delivery via nebulization.

Key words: *Proliposomes, Film deposition on carrier method, Water-soluble carriers, Twin stage impinger (TSI), Nebulization*

INTRODUCTION

In pulmonary drug delivery systems, aerosol-based formulations are preferable for the treatment of respiratory disorders, either localized or systemic or other immune disorders. Due to the large surface area, i.e., 80 to 100 m^2, the pulmonary system allows rapid absorption of drugs into the systemic circulation [1-3]. However, deep deposition in the lungs, in conjunction with improved treatment outcomes, is difficult to achieve. As a result, pulmonary formulations and devices are constantly being researched. Medical nebulizers are devices that produce an inhalable aerosol, allowing for deep lung deposition, effective in targeting, immediate drug release, needle-free administration, and patient compliance [4].

The use of liposomal formulations for the treatment of lung diseases, which are helpful in the entrapment of both hydrophilic and hydrophobic substances, localization, prolonged action, and therapeutic effect, to minimize local irritation and lower systemic side effects [5-9]. Though liposomes for the nebulization method have several applications, they have poor stability, the fusion of vesicles, and drug leakage [3, 4, 10]. As an alternative to liposomes, prolipsomes can be used to increase the stability. Prolipsomes are phospholipid-based preparations that are uniformly distributed over a carbohydrate-based carrier. To deposit the liposomes in the alveoli or lower

respiratory tract, their size should be <5 μm, which can be obtained by proliposome hydration and nebulization. Otherwise, >5 μm particles will be deposited in the trachea (upper respiratory tract) [10-14].

Favipiravir is a modified pyrazine analogue and repurposed drug that targets RNA polymerase (RdRp) enzymes. It is used to treat influenza, Lassa virus, Ebola virus, and COVID-19. At present, only the oral route is available in the form of tablets in higher doses [15]. Favipiravir proliposome dry formulations were developed via the thin-film hydration method, which produces inhalable liposomal aerosol by nebulization, and were characterized and evaluated in the current studies, i.e., *in-vitro* drug release and *in-vitro* drug deposition studies. This is the first proposed study for favipiravir proliposome formulation to target the respiratory tract via nebulization by utilizing phospholipids and carbohydrate carriers.

MATERIALS AND METHODS

Materials

Favipiravir gift sample from Hubert drugs, Hyderabad, India; LECIVA-DPPC gift sample obtained from VAV Lipids, Mumbai, India; Lipoid-S-100 gift sample obtained from Lipoid, Germany; Cholesterol was purchased from Yarrow Chemicals, Mumbai, India; Pearlitol SD- 200 was purchased from Signet chemical Corp., Mumbai, India; Smart EX-QD-100 gift sample from Arihant Innochem Pvt. Ltd., Galen IQ-721 gift sample obtained from Bento palatinit GmbH, Germany.

Preparation of proliposomal powder

The film deposition on carrier method was used for the research of the favipiravir proliposomal formulation as per the procedure mentioned in Naseeb *et al*., [16] The proliposomal powder is formulated with lipid and cholesterol used in combination (1:1 molar ratio) as the lipid phase and water soluble-carrier were dissolved in chloroform and ethanol (8:2% v/v) solvent mixture **(Table 1)**. A rotary flash evaporator (Superfit/ Supervac) is used to remove the organic solvent mixture for 1 hour at 60 °C at 100 revolutions per minute. The resulting proliposomal powder was then stored overnight in a vacuum chamber to remove any remaining solvent. The proliposomal powder product was kept in glass vials and stored at 4°C till further use for characterization and other subsequent studies [16-18].

Table 1. Composition of proliposome formulations

Material (mg)	FPL 1	FPL 2	FPL 3	FPL 4	FPL 5	FPL 6
FVP	40	40	40	40	40	40
CHOL	38.6	38.6	38.6	38.6	38.6	38.6
DPPC	73.4	73.4	73.4	-	-	-
SPC	-	-	-	64.39	64.39	64.39
MSD	100	-	-	100	-	-
SQD	-	100	-	-	100	-
GIQ	-	-	100	-	-	100
Chloroform : Ethanol (8:2)	Up to 10 ml					

Note: FPL 1-3: DPPC; FPL 4-6: SPC; FVP: Favipiravir; CHOL: Cholesterol; DPPC: Di Palmitoyl Phosphatidyl Choline; SPC: Soy Phosphatidyl Choline (Lipoid S-100); MSD: Mannitol Spray Dried (Pearlitol SD- 200; SQD: SmartX QD-100; GIQ: Galen IQ; mg: milligram; ml: milliliter;

Evaluation of favipiravir proliposomes

The prepared favipiravir proliposomal formulations were evaluated for physical appearance, percentage yield, % entrapment efficiency (% EE), % drug content, powder flow properties [19], microscopic examination, hydration study for generation of liposomes, and the number of vesicles per cubic meter procedure as mentioned in Naseeb *et al*., [16].

In-vitro diffusion studies

For determination of drug release from proliposomes, i*n-vitro* drug release studies were done in an open-end boiling tube using a dialysis bag in 200 ml of phosphate buffer 6.8 pH release medium. Continuous magnetic stirring at 100–200 rpm kept the temperature at 37±0.5˚C. For the release study, the proliposome formulation equivalent to 5 mg of favipiravir drug was dispersed in 1-2 ml of phosphate buffer 6.8 pH solution. At predetermined intervals, 1 ml of sample was withdrawn for up to 9 hours, and sink conditions were maintained.

After suitable dilutions, the concentration of samples was determined by UV-spectrophotometry at 237 nm. To understand the barrier effect of the dialysis membrane, the experiment was carried out in triplicate [16, 20].

Model dependent kinetics

To analyze the *in-vitro* release data several kinetic models were used to describe the release kinetics. The zero-order rate, first-order rate, Higuchi, Korsmeyer-Peppas plots, describes the systems where the drug release order and mechanism [19].

Characterization of favipiravir proliposomes

Diffraction scanning calorimetry (DSC)

The thermal investigation for favipiravir pure drug, optimized proliposome formulation were performed using DSC (Shimadzu, Tokyo, Japan). The sample of about 3 to 5 mg was taken each and was placed in sealed standard aluminum pans before heating under a nitrogen flow (50 ml/min) and setting the heat flow from 0 to 300 °C. This study helps in the determination of physical nature [21, 22].

X-ray powder diffraction (XRD)

For determination of phase identification, powder characteristics like crystalline or amorphous were done by XRD studies by X-Ray diffractometer (Shimadzu-Maxima, Japan) Here pure drug and proliposomes powder samples were characterized and compared [23].

Surface morphology by Scanning electron microscopy (SEM)

SEM study was conducted for the surface morphology study. On aluminum stubs, the proliposome powder was sprinkled uniformly. By using a sputter coater, the surface was gold-coated and then images were captured using a scanning electron microscope (Hitachi S-3700N, Japan) [16, 24, 25].

Size and Zeta potential

Dynamic light scattering (Malvern Zeta-sizer) is employed to determine the vesicle size and zeta potential of proliposomes. This study was conducted after the hydration of proliposomes [16, 25].

Liposomes generation from proliposomes by nebulization

For the liposomes generation from proliposomes, 5 mg equivalent of drug containing proliposomal powder formulation, was transferred into the nebulizer reservoir (Optimo- NEB-102 model, Compressor nebulizer) containing phosphate buffer solution or 0.9% w/v normal saline/water for injection, and manual shaking for 3 minutes for the formation of liposomes from proliposomes [26].

In-vitro lung deposition studies or nebulization studies via twin stage impinger

Nebulization studies are helpful to assess the deposition of favipiravir-loaded liposomes during nebulization. For this, a homemade twin stage impinger (TSI-Apparatus-‘B’; European Pharmacopeia, 2004) was used [27]. The twin-stage impinger has three collection stages as well as a mouthpiece to which the nebulizer is attached. The stages 0 and 1 (upper stages) of TSI represent the upper airways of the respiratory tract, and the stage 2 (lower stage) of TSI represents the lower respiratory tract airways. The Cut-off aerodynamic diameter is 6.4 µm at 60 l/min negative pressure by a vacuum pump. Hence the particles less than the cut-off size can deposit in the stage-2 (lower stage) of TSI and are considered as 'fine particle fraction (FPF) or respirable fraction (RF)’ [28-32].

For the *in-vitro* nebulization studies, add 7 ml of phosphate buffer pH 6.8 to the upper stage and add 30 ml of phosphate buffer pH 6.8 to the lower stage of TSI. To the nebulizer reservoir (Stage-‘0’), favipiravir loaded proliposomal powder equivalent to 5 mg of drug dispersed in 5ml of phosphate buffer solution or 0.9 % w/v of normal saline/ water for injection attached to the mouthpiece of the twin stage impinger. The nebulization process continued till dryness to ensure liposomal suspension was finished in the reservoir. Post nebulization, the solutions from stages ‘0’, ‘1’, and ‘2’ were collected and diluted with phosphate buffer and analyzed using UV at 237 nm. The parameters such as nebulization time (min.), the nebulization efficiency (NE) or % mass output (1), % respirable fraction (2), aerosol output rate (mg/min.) (3), etc., were determined by the below given equations. These parameters are helpful to determine the aerodynamic properties of proliposomes via nebulization [33-36].

$$\text{Aerosol mass output or Nebulization efficiency (\%)} = \frac{\text{Aerosolized drug mass deposited in Stage} - 1 \text{ and Stage} - 2}{\text{Weight of the drug mass loaded in nebulizer reservoir (Stage} - '0')} \times 100 \quad (1)$$

$$\text{Aerosol output rate(mg/min.)} = \frac{\text{Aerosolized drug mass deposited in Stage} - 1 \text{ and Stage} - 2}{\text{Complete Nebulization time (Min.)}} \times 100 \quad (2)$$

$$\text{Respirable fraction (\%)} = \frac{\text{Aerosolized drug mass deposited in Stage} - 2}{\text{Weight of the drug mass loaded in nebulizer reservoir (Stage}-'0')} \times 100 \quad (3)$$

As a measure of the performance of nebulized aerosols, the TSI study is a standard model for analysis to determine the nebulization efficiency official method by the British Pharmacopeia (BP), European Pharmacopeia (EP), and the United State Pharmacopeia (USP) [32].

Characterization of liposomes after nebulization

Post nebulization the collected liposomal samples from the stage-0, stage-1, and stage-2 of TSI and diluted accordingly are characterized for vesicle size (Malvern zeta-sizer, Malvern instruments Ltd., UK) and were also analyzed for morphological identification by SEM (Hitachi S-3700N) [29, 37].

Stability studies

Prepared prolipsomes were studied for stability. Optimized formulation packed in an amber colored glass bottle, kept in a desiccator at room temperature and a refrigerator temperature (4 ± 2 °C) for one month. At periodic time intervals, the sample was collected and analyzed for the physical study, hydration study, % entrapment efficiency, drug content, and *in-vitro* drug release.

RESULTS AND DISCUSSION

Characterization of favipiravir proliposomal formulations before nebulization

The favipiravir proliposomes were prepared and analyzed for physical appearance, % yield, % EE, powder flow properties, % drug content, and the number of vesicles per cubic meter data presented in **Table 2.**

Table 2. Results of favipiravir proliposomal formulations

Formulation Code	Physical Appearance	% Yield*	%EE*	% Drug content*	Angle of repose (ɵ)*	Bulk density (gm/cm3) *	Tapped density (gm/cm3) *	Carr's index (%)*	Hausners ratio*	*No. vesicles per m^3 X 10^3
FPL1	Sticky White	81.75±0.05	89.12±0.03	95.31 ± 0.25	35.03±0.015	0.11±0.11	0.125±0.18	12±0.11	1.136±0.17	3.33±0.05
FPL2	Sticky White	80.16±0.03	88.21±0.05	96.07 ± 0.27	35.37±0.09	0.1±0.12	0.125±0.11	20±0.12	1.25±0.22	3.11±0.03
FPL3	Sticky White	81.75±0.02	86.25±0.06	94.3 ± 0.94	34.60±0.055	0.11±0.10	0.13±0.16	15.38±0.22	1.18±0.14	3.07±0.08

FPL4	Free flow yellow powder	80.69±0.04	92.36±0.05	94.14 ± 0.07	31.45±0.16	0.1±0.16	0.11±0.19	9.09±0.15	1.1±0.19	2.99±0.10
FPL5	Free flow yellow powder	79.45±0.03	91.16±0.09	95.47 ± 0.06	34.60±0.18	0.09±0.14	0.11±0.12	15.38±0.18	1.18±0.21	3.12±0.03
FPL6	Free flow yellow powder	79.04±0.02	91.99±0.04	96.33 ± 0.04	31.09±0.26	0.1±0.12	0.11±0.20	9.09±0.20	1.1±0.18	3.13±0.07

Note: % EE= Percentage entrapment efficiency; % Yield = Percentage yield; *Data are mean ±SD, *n*=3;

The % entrapment efficiency of all 6 proliposomal formulations was found in the range of 86.25±0.06 and 92.36±0.05. The results indicate that the formulations comprising SPC lipid and cholesterol with a 1:1 ratio have good entrapment efficiency, this may be due to an increase in hydrophobicity.

The % drug content of all six formulations ranged from 94.14 ± 0.07 to 96.33 ± 0.04, among them, FPL2 and FPL6 formulations have high drug content, which designates the efficient loading of drug into the liposomal vesicles and in the powder proliposomes drug is dispersed uniformly. The SPC-based proliposomal formulations have good flow properties due to the type of lipid and carrier [16].

Hydration study

On hydration, the conversion of liposomal vesicles formation from proliposomes was observed in 1 to 3 minutes. The quick modification confirms that proliposomes readily coverts to liposomes on interaction with the water or physiological fluid [11, 16].

In-vitro diffusion studies

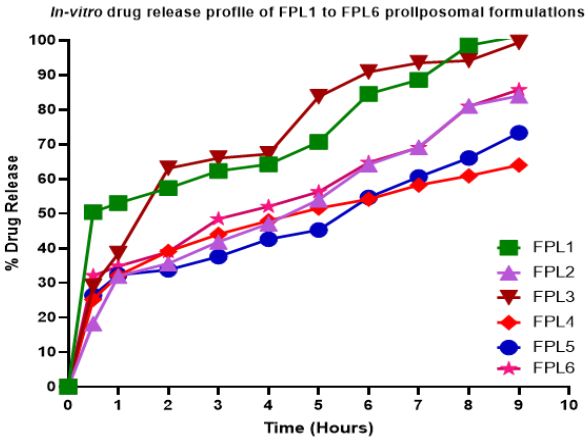


Figure 1. *In-vitro* drug diffusion profile of favipiravir proliposomal formulations (FPL1 to FPL6)

For FPL6 proliposomal formulation containing SPC and GIQ as the carrier has a % of drug release of 86.20±0.66% for 9 hours in 6.8 pH phosphate buffer due to lipid and carrier [16, 20]. The dissolution profile of proliposomal formulations (FPL1 and FPL6) was shown in **Figure 1**. Based on flow properties, %EE and *in-vitro* drug release studies, the FPL6 proliposomal formulation was selected for further characterization.

Model dependent kinetics of FPL6 formulation

The optimized proliposomal formulation FPL6 was fit into mathematical equations and was concluded that drug release followed first-order kinetics (r^2 =0.933) and Higuchi model (r^2 =0.964) with anomalous diffusion release mechanism (n=0.759) [19].

Diffraction scanning calorimetry (DSC)

The DSC thermogram of favipiravir (pure drug), optimized proliposomal formulation (FPL6). DSC studies revealed that there is no significant shift in the melting point and there is no endothermic peak in the FPL6 formulation. The halo pattern denotes amorphous nature [22].

X-ray powder diffraction (XRD)

The XRD studies of favipiravir (pure drug), optimized proliposomal formulation (FPL6). XRD studies revealed that the favipiravir drug is crystalline in nature and the FPL6 proliposomal formulation is semi-crystalline in nature or amorphous in nature [23].

Surface morphology by SEM before nebulization

In SEM characterization, the optimized proliposomal formulation FPL6, exhibited as irregular, coarse, non-porous, in clumps and had no distinct edges **Figure 2** [24].

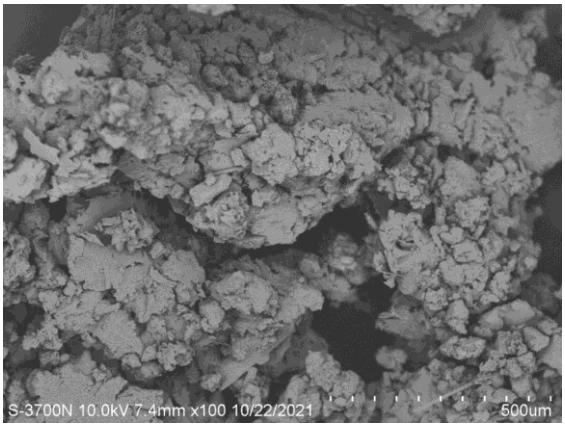


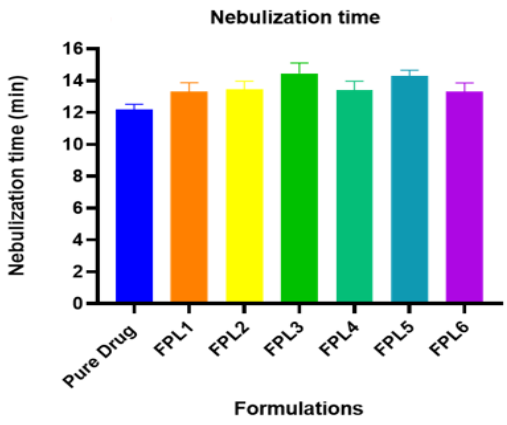
Figure 2. SEM image of FPL6 proliposomal formulation (Pre Nebulization)

Size and Zeta potential

The optimized formulation FPL6 showed the mean size of 373.2 nm, with a polydispersity index (PDI) of 0.781 indicating a uniform size of liposomal vesicles and zeta potential was -36.2 mV. The negative charge may be due to the negative charges of the polar head groups of the phospholipid. The zeta potential value designates the prevention of vesicles aggregation and improved stability [16, 18].

In-vitro lung deposition studies or nebulization studies via twin stage impinger

The nebulization time was between 12.95 and 14.3 minutes **(Figure 3a)**; the aerosol mass output (%) was between 44.31 and 54.28 percent **(Figure 3b)**; the aerosol output rate (mg/min) was between 0.17 and 0.22 percent **(Figure 3c)**, and the respirable fraction (%) was between 8.14 and 12.31 percent **(Figure 3d)**. The FPL6 formulation has the highest respirable fraction of 12.31 % was due to smaller sized vesicles formation and rapid hydration [23, 29, 32].



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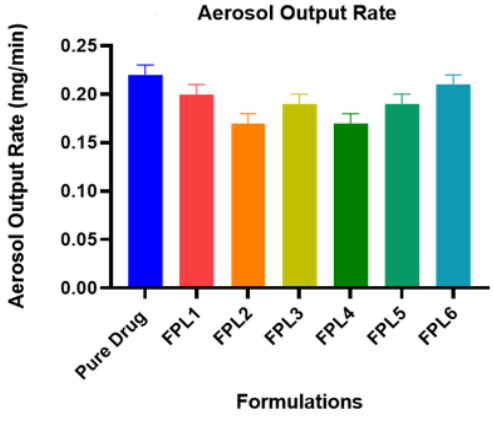

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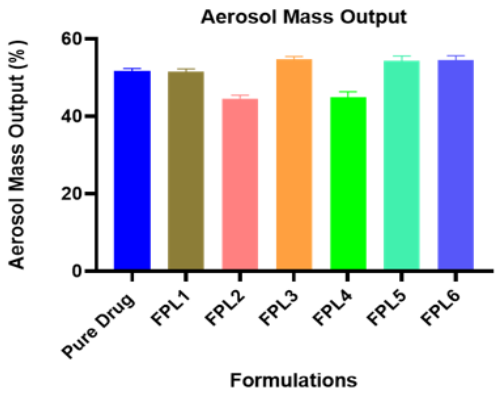

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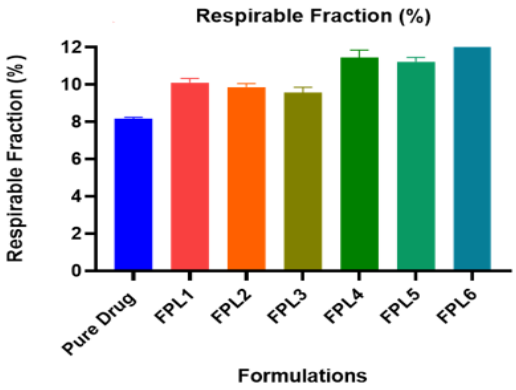
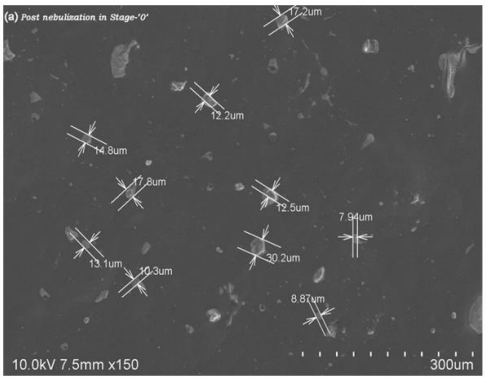


d)

Figure 3. Post nebulization studies results of FPL6 proliposomal formulation (a) Nebulization time (b) Aerosol mass output (%) (c) Aerosol output rate (mg/min) (d) Respirable fraction (%)

Characterization of liposomes after nebulization

Post nebulization FPL6 proliposomal formulation in Stage-0, Stage-1, and Stage-2 of TSI was studied for surface morphology **(Figures 4a-4c)** and vesicle size. Post nebulization, the vesicle size was reduced and deposited in stage 2 appears to be smaller micron size in both stages [23, 29, 32].



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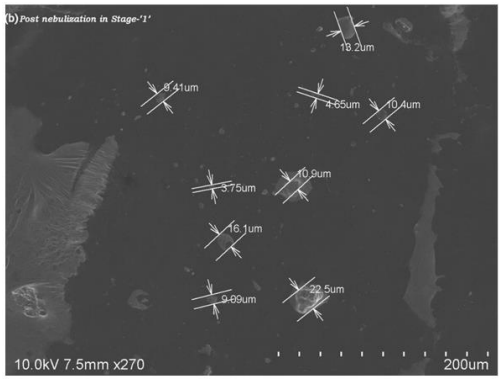

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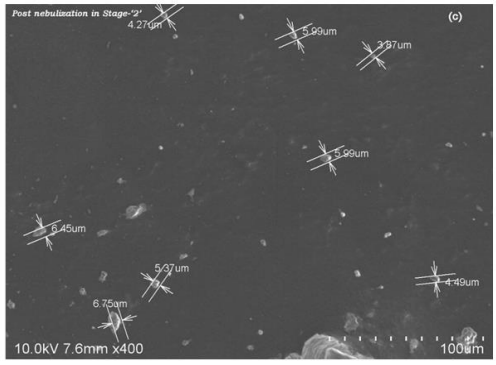

c)

Figure 4. SEM images of FPL6 proliposomal formulation post nebulization (a) Stage-'0' (b) Stage-'1' (c) Stage-'2'in TSI

Stability studies

Stability studies were performed for the optimized favipiravir proliposomal formulation (FPL6). There were no significant changes observed during the storage and the product was stable at 4 °C for 1 month.

CONCLUSION

A proliposomal vesicular drug delivery system for favipiravir was formulated using the film deposition on carrier method and evaluated for pulmonary delivery by nebulization. *in-vitro* diffusion studies and in-*vitro* lung

deposition studies by TSI and other studies revealed that the formulation is stable and suitable for administration by nebulization. Hence, this formulation is suitable for pulmonary delivery and could be used for scale-up. Further *in-vivo* lung deposition studies in animals need to be conducted to prove the deposition of favipiravir in the lungs.

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