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Tenofovir Alafenamide Fumarate Microspheres, Process Parameters for Enhanced Permeability and Liver Targeting

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: Chronic hepatitis B (CHB) infection is a serious global health problem and one of the main causes of chronic liver disease, cirrhosis. Tenofovir Alafenamide Fumarate (TAF) is a prodrug of Tenofovir, a nucleotide analogue with limited oral bioavailability. TAF is considered to be a BCS Class III substance (high solubility, low permeability). The aim of the study was to develop the Tenofovir Alafenamide Fumarate (TAF) microspheres to improve permeability.
Study design: Preparation and Evaluation of Microspheres.
Place and Duration of Study: Department of pharmaceutics, Mallareddy Institute of Pharmaceutical Sciences, Affiliated to JNTUH, Hyderabad, Telangana, India, between January 2018 and June 2019.
Methodology: TAF loaded chitosan microspheres were prepared by emulsion cross linking method using glutaraldehyde as cross-linking agent. The prepared microspheres were characterized by morphology, size distribution, encapsulation efficiency. The permeability study

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was evaluated by Ex-vivo permeation studies. The optimized formulation was subjected to FTIR studies to examine the Drug Excipient Compatibility.
Results: Scanning Electron Microscopy (SEM) studies indicated that the microspheres are spherical in shape. The optimized formulation has average particle size of 11.00 ± 0.05 µm, the encapsulation efficiency 68
± 0.04% and the percentage (%) yield of 94%. FTIR studies indicated that the drug and polymer are compatable with each other. In Ex-vivo permeation studies of optimized formulation 80% of drug was permeated with in 60 min.
Conclusion: TAF microspheres could improve the absorption by increasing the permeability.

Keywords: Microspheres; emulsion cross linking; glutaraldehyde and Tenofovir Alafenamide Fumarate (TAF).

1. INTRODUCTION

Globally, chronic hepatitis B virus (HBV) infection is a crucial trending healthcare issue because of the increased risk of liver-related morbidity and mortality associated with persistent viraemia [1]. Chronic hepatitis B (CHB) infection is a serious global health problem and one of the main causes of chronic liver disease, cirrhosis and hepatocellular carcinoma (HCC). It has been estimated that 250–350 million individuals are positive to hepatitis B surface antigen (HBsAg), yielding a worldwide prevalence of 3.6%, with considerable geographic variability. Currently, there are two options for treating CHB: one is interferons and the other is oral antiviral agents. Of these, treatment with oral antiviral agents has been more successful in achieving maintained viral suppression in CHB patients, an effect associated with a decrease in long-term complications.

Tenofovir alafenamide fumarate (TAF) is a phosphonamidate prodrug of tenofovir, a nucleotide analogue with limited oral bioavailability that inhibits HBV and human immunodeficiency virus type 1 (HIV-1) reverse transcription. Tenofovir Alafenamide Fumarate (TAF), a new prodrug of tenofovir and a potential successor of tenofovir disoproxil fumarate (TDF), has been approved in the United States and Europe for treating adolescents and adults with chronic hepatitis B infection. Tenofovir alafenamide act by entering into primary hepatocytes by passive diffusion and by the hepatic uptake transporters OATP1B1 and OATP1B3, and is then hydrolyzed by carboxylesterase 1 to form Tenofovir [2]. This is then phosphorylated to Tenofovir diphosphate. Incorporation of tenofovir diphosphate into HBV DNA by HBV reverse transcriptase inhibits HBV replication, leading to DNA chain termination.

Over the past many years, encapsulation of active chemical ingredients has attracted great deal of attention because of their potential application in pharmaceutical industry. It offers greater effectiveness, lower toxicity, lasting stability and improved patient compliance. Various methods are available for preparation of microspheres but choice of particular methods mainly determined by drug solubility and molecular stability concerns [3].

Chitosan [(1→4) 2 amino 2-deoxy-b-D-glucan] is a polyaminosaccharide, typically obtained by alkaline deacetylation of chitin, which is a very abundant naturally occurring polymeric material (Yao et al. 1995). Chitosan has attracted attention as a matrix for controlled release, since it possesses reactive functionalities, is easily degraded by enzymes and the degradation products are non-toxic [4]. Chitosan, being a cationic polysaccharide in neutral or basic pH conditions, contains free amino groups and hence, is insoluble in water. In acidic pH, amino groups can undergo protonation thus, making it soluble in water. Usually 1–3% aqueous acetic acid solutions are accustomed to solubilize Chitosan. Chitosan is biocompatible with living tissues since it doesn't cause hypersensitive reactions and rejection. It breaks down slowly to harmless products, which are completely absorbed by the human body [5]. The positive charge on chitosan, generated under physiological conditions, has been found to be responsible for its enhanced bio adhesion to negatively charged cell membranes. This enables the site-specific applications in controlled delivery systems [6,7,8].

For the preparation of chitosan microspheres glutaraldehyde is most commonly used cross linking agent. In emulsion cross linking method reaction of chitosan with aldehyde groups often forms covalent imine bonds with the amino groups of chitosan. This is due to the resonance established with adjacent double ethylenic bonds via a Schiff reaction. In this method the size of particles can be controlled by controlling the size of aqueous droplets [5,9].

Literature revealed that, liver targeting efficiency of microspheres lying in the size range of 5-25 µm is very high [10] and especially the microspheres of 13 µm shown significant higher concentration in the liver compared to other organs [11].

Therefore, the main objective of the present work is to develop the TAF loaded chitosan microspheres to improve the absorption and the permeation to achieve maximum accumulation in liver by assessment particle size and encapsulation efficiency. The enhanced permeation was evaluated by *ex-vivo* diffusion studies.

2. MATERIAL AND METHODS

Tenofovir Alafenamide Fumarate was obtained as gift sample from Mylan Laboratories Pvt. Ltd. Chitosan was purchased from Yarrow chemical products, Mumbai, India. Liquid paraffin and N-Hexane were obtained from Finar, Mumbai, India. Tween 80 was obtained from BDH Laboratory Supplies, England. Acetone was purchased from Fischer scientific, Germany. All the chemicals were of analytical grade.

2.1 Methods

2.1.1 Preparation of Chitosan Microspheres Loaded with TAF

Chitosan microspheres containing TAF were prepared using an emulsion cross-linking technique. Fourteen trial formulations, F1-F14 were tried and their composition is given in Table 1. Chitosan was dissolved in 2% v/v acetic acid solution. Drug was dissolved in water. Drug solution was added to chitosan solution and mixed thoroughly. The mixture was then degassed in a sonicator for 10 minutes. 100 ml of light liquid paraffin containing 0.5% v/v Tween 80 was then poured into a 250 ml beaker and stirred with a mechanical stirrer. The chitosan-drug solution was added ndrop-wise, under stirring, to form a w/o emulsion. After 20 min, 25% v/v glutaraldehyde (cross linking agent) was added stage by stage. Stirring continued for 3 hours until the microspheres were obtained. They were then decanted from the paraffin and washed 3 times with n-hexane, followed by acetone, to remove the paraffin oil [12,13].

2.2 Characterization of Drug-Loaded Microspheres

2.2.1 Morphology and Size of Microspheres

The surface structure, size and size distribution of the chitosan microspheres were characterized from the micrographs taken with the scanning electron microscope (ZEISS scanning electron microscope) and optical microscope. In the SEM studies; a 100 µl aqueous suspension of microspheres was dried on a metal support in vacuo at room temperature for 4 h and the samples were coated with gold. The size and size distribution of the chitosan microspheres were determined by using optical microscopy.

2.2.2 Encapsulation Efficiency

Microspheres (10 mg) were ground in a mortar, and added to water in 10 ml volumetric flask and then sonicated for 20 min and kept aside for 24 hrs. The supernatant was centrifuged to remove the polymeric debris and then measured at 261 nm with UV spectrophotometer.

The drug encapsulation efficiency was calculated using the following formula:

$$\% \text{ Encapsulation efficiency} = \frac{\text{Actual quantity of drug determined}}{\text{Theoretical quantity of drug}} \times 100$$

2.2.3 Percentage Yield

Thoroughly dried microspheres were collected and weighed accurately. Percentage yield was calculated as the ratio of the mass of microparticles and the mass of initial substances added, including the drug and polymer.

$$\text{Percentage Yield} = \frac{\text{Weight of microspheres}}{\text{Total expected weight of drug and polymer}} \times 100$$

Table 1. Composition of TAF microspheres (Quantities Reduced to Single Dose)

Formulation Code	Chitosan (mg)	Drug (mg)	Glacial Acetic Acid solution (ml)	Chitosan / Glacial AceticAcid SolutionRatio	Stirrer(S) / Homogenizer (H)	Speed (rpm)
F1	25	25	25.00	1	S	1000
F2	25	25	12.50	2	S	1000
F3	50	25	12.50	4	S	1000
F4	75	25	12.50	6	S	1000
F5	25	25	6.25	4	S	1000
F6	50	25	6.25	8	H	1000
F7	25	25	2.50	10	H	1000
F8	50	25	5.00	20	H	1000
F9	25	25	2.50	10	S	1000
F10	50	25	5.00	20	S	1000
F11	25	25	2.50	10	H	1500
F12	25	25	2.50	10	S	1500
F13	25	25	2.50	10	H	2000
F14	25	25	2.50	10	S	2000

2.2.4 FT-IR Spectroscopy

FT-IR studies were conducted to know the chemical reaction between TAF and chitosan. The spectra of TAF, chitosan, TAF-loaded microspheres were recorded on FT-IR spectrophotometer by KBr. pellet method. (Perkin Elmer), USA). All of the pellets were prepared by crushing samples with KBr. The pellet was placed in a sample holder and spectral scanning was taken in a wave length region between 4000-500 cm^{-1} with a speed of 1 cm/s.

2.2.5 Ex-vivo Permeation Studies by using Goat Intestinal Membrane

The permeability study was conducted using Franz diffusion cell. The Franz cell is a diffusion chamber, made of glass comprising of donor compartment, and receptor (acceptor) compartment.

The receptor compartment was filled with phosphate buffer pH 7.2. Between the compartments, the microspheres sprinkled on tissue was clamped with the mucosal side oriented upwards (Singh et al., 2010). The hooks were secured with rubber bands on the sides of both compartments. The fluid, in the receptor compartment, was maintained at 37 ± 0.5ºC and stirred continuously at 50 rpm, using thermostatically controlled magnetic stirrer with Teflon coated bead. The external jacket of Franz diffusion cell was connected to a water bath so as to maintain temperature in cell.

Aliquot (1 ml) was withdrawn periodically at preset time from the above- mentioned receiver cell, which was diluted and TAF content was determined by UV spectrophotometer at 261 nm. The volume of withdrawn sample was replaced by same volume of buffer to keep the volume constant so that sink condition could be maintained. Experiment was carried out upto 12 h with the excised intestinal mucosal membranes.

3. RESULTS AND DISCUSSION

3.1 Preparation of Chitosan Microspheres

The prepared microspheres of Tenofovir Alafenamide Fumarate were obtained in brown colour. In F1 and F2 the particles are not formed which may be due to less proportion of chitosan in acetic acid.

3.1.1 Effect of Stirring Rate

Emulsifier concentration, chitosan to acetic acid solution ratio and glacial acetic acid concentration were kept constant and the stirring speed was changed between 1000 to 2000 rpm. The average size of the chitosan microspheres decreases and the size distribution becomes narrower when the stirring rate is increased. This may be due to higher stirring rate provides required energy to chitosan solution to be dispersed as a fine droplet in oil phase.

3.1.2 The Effect of Chitosan/Acetic Acid Solution Ratio

The Chitosan to Acetic acid solution ratio was varied between 1-20. The average size and size distribution of Chitosan microspheres increased by increase in the Chitosan to Acetic acid solution ratio.

3.2 Morphology and Size of Microspheres

The particle size of microspheres was determined by using optical microscopy technique. The optical microscopy image of microspheres under 40 X was shown in Fig. 1. SEM image of microspheres was shown in Fig. 2. SEM image revealed that the individual particles are produced with irregular outer surface and are almost spherical in shape.

As shown in Table 2. formulations F11 to F14 were produced in 11.0 ± 0.05 µm,11.4 ± 0.05 µm, 13.2 ± 0.05 µm, 10.5 ± 0.05 µm indicating that the procedure is successfully used to produce microspheres which can accumulate in liver.

3.3 Encapsulation Efficiency and Drug Content

The encapsulation efficiency of TAF was increased with increase in drug polymer ratio. The increase in drug polymer ratio may leads to the formation of large droplets with decreased surface area. This results in slower diffusion of drugs.

Drug–polymer ratio was kept constant and stirring speed was changed. At lower speeds encapsulation efficiency was more compared to high speeds. This may be due to the formation of large droplets at lower speed. The encapsulation efficiency for optimized formulation (F11) was 68 ± 0.04 %. The encapsulation efficiency for F3-F14 were given in Table 3.

3.4 Percentage Yield

Percentage Yield is more for microspheres prepared by using homogenizer when compared with microspheres prepared with mechanical stirrer, due complete formation of microspheres by homogenization technique. The % yield for optimized formulation was (F11) 94. The percentage (%) yields for F3-F14 were given in Table 3.

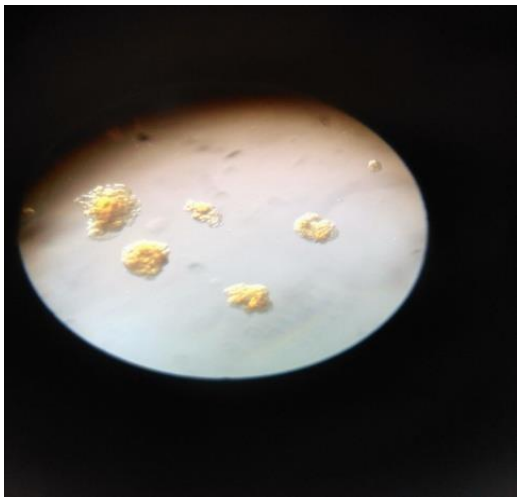

Fig.1. Optical Microscopy Image

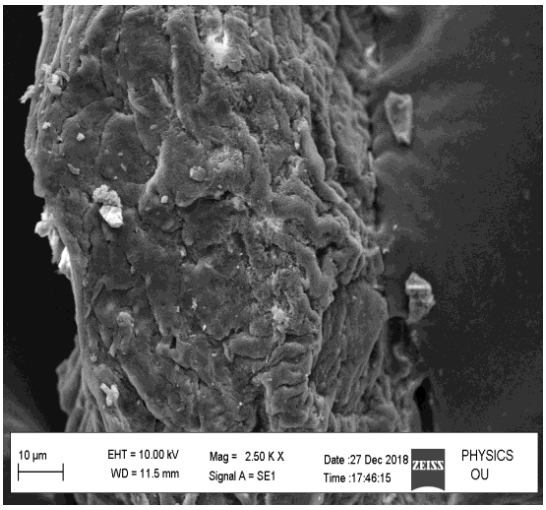

Fig. 2. Scanning Electron Microscopy (SEM) image

Table 2. Average Particle Size of Formulations

Formulation Code	Average Particle Size (µm) {n=3±S.D}
F3	92.7 ± 0.01
F4	107.1 ± 0.05
F5	71.0 ± 0.07
F6	53.5 ± 0.04
F7	23.6 ± 0.02
F8	45.3 ± 0.01
F9	33.0 ± 0.20
F10	49.0 ± 0.01
F11	11.0 ± 0.05
F12	11.4 ± 0.05
F13	13.2 ± 0.08
F14	10.5 ± 0.10

Table 3. Encapsulation efficiency and percentage (%) yield of formulations

Formulation Code	% Encapsulation efficiency	% Yield
F3	48.2 ± 0.03	54.2
F4	57.4 ± 0.07	48
F5	61.2 ± 0.05	57.4
F6	65.3 ± 0.08	78.1
F7	71 ± 0.12	89.6
F8	67 ± 0.05	76
F9	71 ± 0.1	72.0
F10	55 ± 0.03	76.0
F11	68 ± 0.04	94.0
F12	63 ± 0.06	79.0
F13	59 ± 0.01	90.0
F14	57 ± 0.2	73.0

3.5 FT-IR Spectroscopy

FT-IR spectra of TAF, chitosan and TAF loaded microspheres were compared. In FTIR spectrum (Fig. 3) of TAF peak at 1745.4 cm^{-1} was related to ester C=O stretch. The same peak was observed in optimized formulation (Fig. 4) at1736 cm^{-1}. In TAF spectrum the peak at 1301 was related to Acyl C-O stretch. The same peak was observed in optimized formulation at 1302. The spectrum of chitosan (Fig. 5) showed peak at 1642.5

due to amine group. The spectrum of optimized formulation showed peak at 1642.5 and 3359 it may be due to C=N stretching vibration when amine group of chitosan reacts with glutaraldehyde. From the FTIR spectra it was revealed that chitosan was cross linked with glutaraldehyde through Schiff base reaction. FTIR studies indicated that the drug and polymer are compatable with each other.

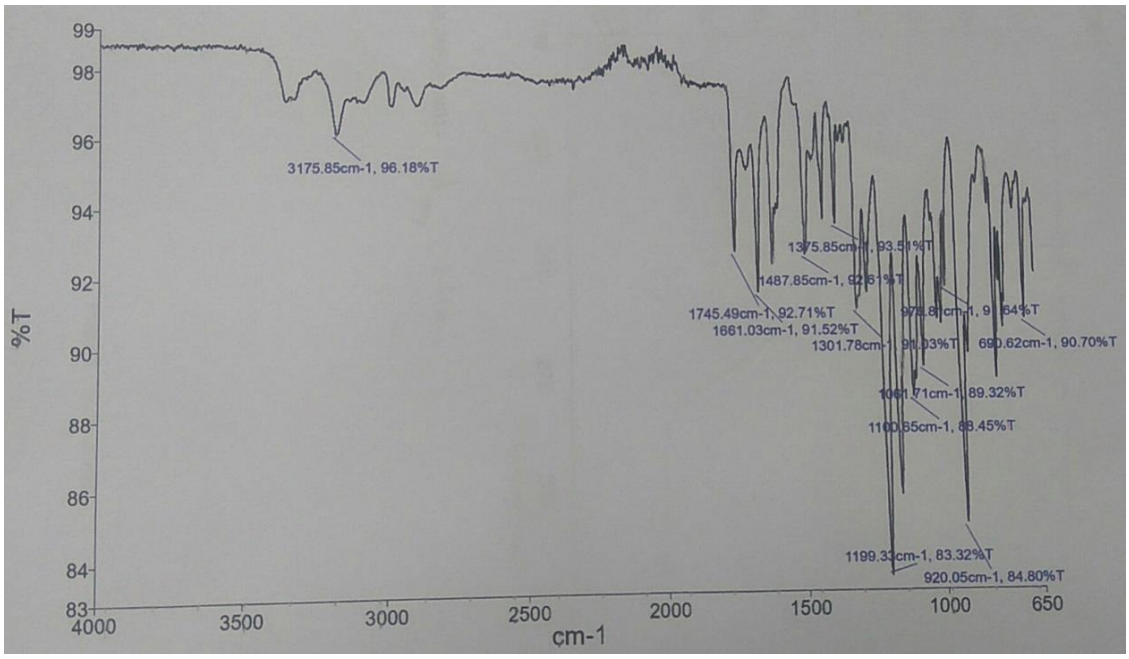

Fig. 3. FT-IR of Tenofovir Alafenamide Fumarate (TAF)

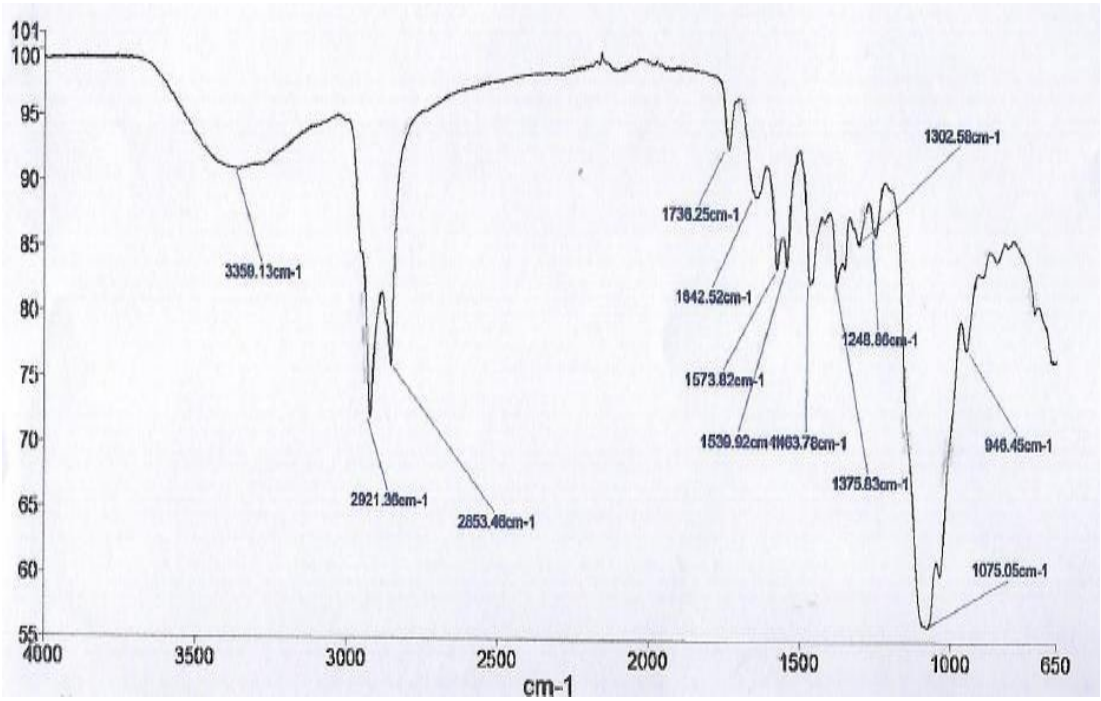

Fig. 4. FTIR of optimized formulation F-11

3.6 *Ex-vivo* permeation studies by using goat intestinal membrane

The *ex-vivo* permeation studies by using goat intestinal membrane for formulations F3-F8 were given in Table 4 and Fig. 6. Formulations F9-F14 were given in Table 5 and Fig. 7 respectively. The amount of drug permeated through goat intestine from different formulations depends on the concentration of polymer. The rate of permeation of drug from microspheres was decreased with increase in polymer concentration. This may be due to higher polymer concentration leads to large particle size which in turn takes longer time for diffusion of drug. For the optimized formulation 80% of drug was permeated with in 60 mins.

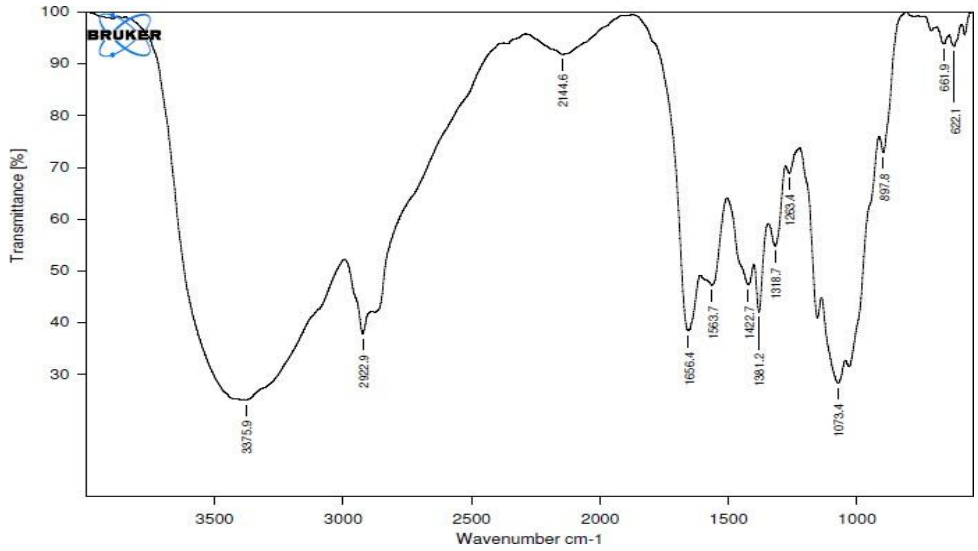

Fig. 5. FTIR of Chitosan

Table 4. *Ex-vivo* permeation studies by using goat intestinal membrane

Time (Min) / Formulation	% Cumulative drug permeated {n= 3 ± S.D}					
	F3	F4	F5	F6	F7	F8
0	0.0	0.0	0.0	0.0	0.0	0.0
15	15.2 ± 0.5	12.1 ± 0.5	21.9 ± 0.7	24.2 ± 0.8	27.5 ± 0.3	28.6 ± 0.2
30	28.5 ± 2.1	25.4 ± 0.7	33.2 ± 0.8	38.1 ± 0.5	40.2 ± 0.5	44.4 ± 0.3
45	34.2 ± 0.8	39.5 ± 1.2	44.5 ± 2.8	47.3 ± 1.6	56.3 ± 0.6	58.6 ± 0.3
60	40.4 ± 0.8	56.7 ± 0.9	58.2 ± 1.3	64.9 ± 3.2	68.2 ± 2.8	70.2 ± 0.7
90	47.0 ± 1.3	60.9 ± 0.7	62.1 ± 0.5	69.1 ± 1.1	70.3 ± 2.4	72.4 ± 0.7
120	55.6 ± 2.6	66.9 ± 3.8	69.7 ± 0.5	73.4 ± 0.4	72.2 ± 0.9	74.8 ± 1.3
150	63.7 ± 0.3	69.1 ± 0.8	71.2 ± 2.5	78.8 ± 1.7	78.0 ± 0.4	75.6 ± 1.1
180	70.4 ± 1.5	74.8 ± 0.9	72.3 ± 2.1	83.4 ± 1.7	79.2 ± 3.4	76.8 ± 2.5

Table 5. *Ex-vivo* permeation studies by using goat intestinal membrane

Time (Min) / Formulation	% Cumulative drug permeated {n= 3 ± S.D}					
	F9	F10	F11	F12	F13	F14
0	0.0	0.0	0.0	0.0	0.0	0.0
15	18.4 ± 0.4	14.1 ± 0.5	26.2 ± 0.2	24.1 ± 0.3	27.0 ± 0.8	25.5 ± 0.5
30	32.3 ± 0.8	28.2 ± 0.5	43.4 ± 0.5	42.0 ± 0.5	40.2 ± 1.1	39.8 ± 0.6
45	55.2 ± 0.1	39.6 ± 0.2	68.2 ± 0.3	60.1 ± 0.1	62.4 ± 0.6	58.8 ± 0.2
60	62.6 ± 0.4	58.2 ± 0.7	80.2 ± 0.1	78.8 ± 0.6	75.2 ± 0.2	76.6 ± 0.9
90	64.0 ± 1.2	62.1 ± 0.5	88.4 ± 0.8	84.0 ± 1.0	81.4 ± 0.7	84.4 ± 1.3
120	72.2 ± 0.8	68.4 ± 2.5	96.5 ± 1.7	93.1 ± 2.1	92.6 ± 0.3	90.0 ± 1.1
150	74.5 ± 0.9	73.3 ± 2.0	98.1 ± 1.7	93.0 ± 3.5	92.0 ± 2.3	94.2 ±1.5
180	74.6 ± 0.4	73.8 ± 1.8	98.0 ± 2.4	93.0 ± 3.5	92.0 ± 1.3	94.1 ± 0.9

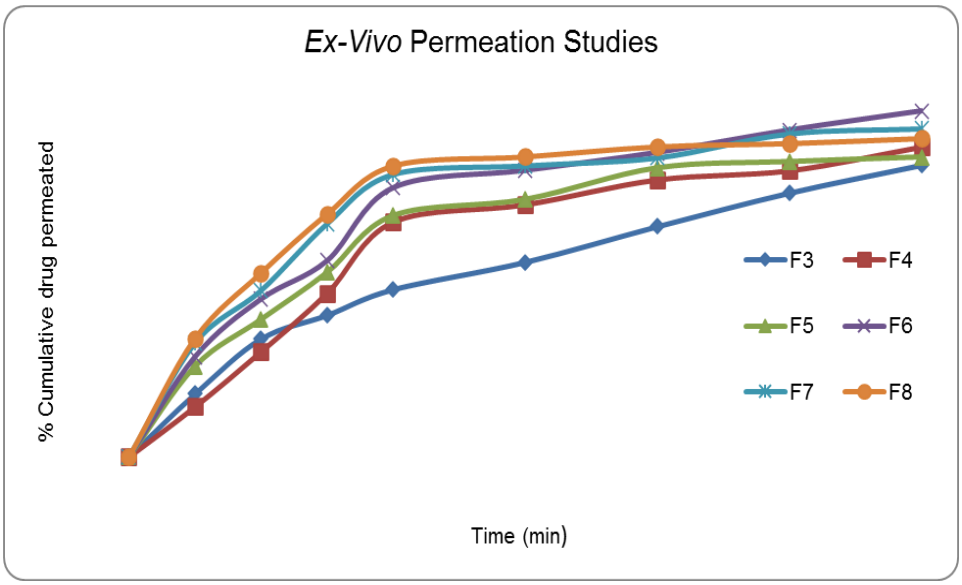

Fig. 6. Ex-vivo permeation studies of formulations f3-f8 by using goat intestinal membrane

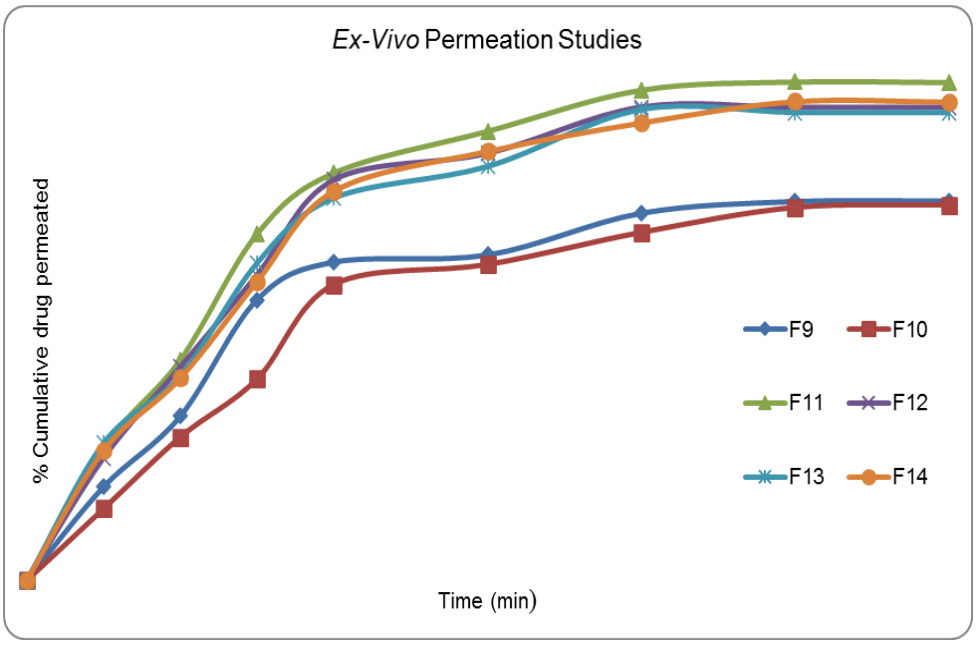

Fig. 7. *Ex-vivo* permeation studies formulations F9-F14 by using goat intestinal membrane

4.CONCLUSIONAND RECOMMENDATION

4.1 Conclusion

Tenofovir alafenamide fumarate microspheres can be produced successfully using chitosan to glacial acetic acid ratio 10 by emulsion cross linking technique with process parameter 1500 rpm using homogenizer with *ex-vivo* permeation of 80% within 60 min and particle size of 11.0 ± 0.05 µm showing its target accumulation in liver to treat Hepatitis B infection.

4.2 Recommendations

In-Vivo studies should be carried out using animal models to investigate the accumulation of drug content in the liver from microspheres.

DISCLAIMER

The company name used for this research is commonly and predominantly selected in our area of research and country. There is absolutely no conflict of interest between the authors and company because we do not intend to use this

company as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the company rather it was funded by personal efforts of the authors.

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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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