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Research Article

DACLATASVIR DIHYDROCHLORIDE MICROSPHERES, PROCESS PARAMETERS FOR ENHANCED PERMEABILITY AND LIVER TARGETING

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ABSTRACT

Objectives: Daclatasavir dihydrochloride (DCLD) is used to treat hepatitis C. DCLD can be used to patients with all stages of compensated liver disease including cirrhosis. The aim of the present study was to develop DCLD microspheres to improve the permeation and maximum accumulation in the liver and *in vitro* evaluation.

Methods: DCLD microspheres were prepared with chitosan polymer using emulsion crosslinking technique. Twelve formulations were prepared, that is, F1-F12. The microspheres were evaluated for morphology, particle size, encapsulation efficiency, % yield, and permeability. FTIR studies were conducted on optimized formulation to check the drug-excipient compatibility.

Results: The particle size of microspheres was in the range of 11.50±0.08 µm to 98.50±0.05 µm. Encapsulation efficiency of the formulations was observed in the range 47.8–69.2%. The *ex vivo* permeation studies revealed that 83.3±0.1% of drug was diffused from microspheres in 60 min, whereas from pure drug 49±0.7% of drug was diffused in 60 min.

Conclusion: DCLD microspheres were shown good permeability when compared to pure drug which will improve the absorption.

Keywords: Daclatasavir dihydrochloride, Emulsion crosslinking technique, Chitosan, *Ex-vivo* permeation studies.

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INTRODUCTION

Hepatitis C is caused by infection with hepatitis C virus (HCV) [1]. HCV infection is a leading cause of chronic hepatitis, liver cirrhosis, and 49 hepatocellular carcinomas. It is estimated that up to 180 million individuals worldwide are chronically infected by HCV [2] Interferon has been the backbone of HCV treatment for the past 10 years, despite the fact that it is still far from perfect. The recent advancement in novel direct antivirals has fundamentally altered how chronic hepatitis C is treated. Inhibitors of the HCV NS5A region have remarkable interest, due to their high potency and favorable safety profile [3]. Daclatasvir is a direct-acting antiviral agent against HCV used for the treatment of chronic HCV genotype 3 infection. Daclatasvir binds to NS5A, a nonstructural phosphoprotein that is encoded by the HCV virus, and stops RNA replication and virion assembly. Binding to the N-terminus of the D1 domain of NS5A prevents its interaction with host cell proteins and membranes required for virion replication complex assembly [4]. Treatment with daclatasvir has yielded high rates of virologic response in patients infected with genotype (Gt) 1 and Gt 3, when used in combination with other antivirals of a different class, such as sofosbuvir [3]. Daclatasvir inhibits the HCV non-structural protein NS5A [5]. It targets two steps of the viral replication process and allows rapid fall of HCV RNA. Daclatasvir is indicated for use with sofosbuvir for the treatment of patients with chronic HCV genotype 2 infections [2]. The aim of present study was to develop the daclatasavir dihydrochloride (DCLD) microspheres to improve the permeation and maximum accumulation in the liver by controlling the particle size and to conduct the *in vitro* studies.

METHODS

Materials

DCLD was obtained as gift sample from Mylan Laboratories Limited (A Viatris company), Hyderabad, India. Chitosan was purchased from Yarrow chemical products, Mumbai, India. Liquid paraffin, glacial acetic acid 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.

Preparation of chitosan microspheres loaded with DCLD

Chitosan microspheres containing DCLD were prepared using an emulsion cross-linking technique. Twelve trial formulations were prepared and named as F1-F12 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 min. 100 mL of light liquid paraffin containing 0.5% v/v Tween 80 was poured into a 250 mL beaker and stirred with a mechanical stirrer. The chitosan-drug solution was added drop-wise while being stirred to form a w/o emulsion. After 20 min, 25% v/v glutaraldehyde (cross linker) was added gradually. Stirring continued for 3 h 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 [6,7].

Characterization of drug-loaded microspheres

Morphology and size of microspheres

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

Encapsulation efficiency

Microspheres (10 mg) were ground in a mortar, added to water in 10 mL volumetric flask, sonicated for 20 min, and kept aside for 24 h.

Table 1: Composition of DCLD microspheres (quantities reduced to single dose)

Formulation code	Chitosan (mg)	DCLD (mg)	Glacial acetic acid solution (mL)	Chitosan/glacial acetic acid solution ratio (mg/mL)	Stirrer (S)/ homogenizer (H)	Speed (rpm)
F1	30	30	15.0	2	S	1000
F2	60	30	15.0	4	S	1000
F3	90	30	15.0	6	S	1000
F4	60	30	15.0	4	H	1000
F5	90	30	15.0	6	H	1000
F6	30	30	7.5	4	S	1000
F7	30	30	7.5	4	H	1000
F8	30	30	7.5	4	H	1500
F9	30	30	3.0	10	S	1500
F10	30	30	3.0	10	H	1500
F11	30	30	3.0	10	S	2000
F12	30	30	3.0	10	H	2000

The supernatant was centrifuged to remove the polymeric debris and then measured at 314 nm with UV spectrophotometer.

The drug encapsulation efficiency was calculated using the following formula:

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

%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.

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

FT-IR spectroscopy

FT-IR studies were conducted to know the chemical reaction between DCLD and chitosan. The spectra of DCLD, chitosan, and DCLD-loaded microspheres were recorded on FT-IR spectrophotometer (Perkin Elmer), USA by KBr. pellet method. 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 and 500 cm^{-1} with a speed of 1 cm/s.

***Ex vivo* permeation studies**

The Franz diffusion cell was used for the permeability investigation. It was made up of glass comprising of donor compartment and receptor (acceptor) compartment. Phosphate buffer with a pH of 7.2 was put into the receptor compartment. Between the compartments, the microspheres sprinkled on tissue were clamped with the mucosal side facing upward. The sidewalls of both compartments had hooks that were fastened with rubber bands. Using a thermostatically controlled magnetic stirrer with a Teflon-coated bead, the fluid in the receptor compartment was kept at 37±0.5°C and continuously swirled at 50 rpm. To keep the temperature in the cell constant, the external jacket of the Franz diffusion cell was linked to a water bath.

A small amount (1 mL) was withdrawn periodically at preset time from the receiver cell, which was diluted and DCLD content was determined by UV spectrophotometer at 314 nm. The volume of withdrawn sample was replaced by same volume of buffer to maintain constant volume so that sink condition could be maintained. Experiment was carried out up to 3 h. with the excised intestinal mucosal membranes.

RESULTS AND DISCUSSION

The microspheres prepared by emulsion crosslinking method were brown in color. In F1, the particles are not formed due to the chitosan to acetic acid solution ratio was less. Emulsifier concentration, chitosan to acetic acid solution ratio, and glacial acetic acid concentration were kept constant and the stirring speed was changed between 1000 and 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 finedroplets in oil phase. The chitosan to acetic acid solution ratio was varied between 1 and 10. The average size and size distribution of chitosan microspheres increased by increase in the chitosan to acetic acid solution ratio.

Morphology and size of microspheres

The particle size of microspheres was determined using optical microscopy technique. SEM image of microspheres is shown in Fig. 1. SEM image revealed that the individual particles are produced with irregular outer surface and are almost spherical in shape. The average particle size of optimized formulation (F10) was 15.70±0.05 μm was presented in Table 2. The average particle size of formulations was in the range of 11.50±0.08 μm to 98.50±0.05 μm. Average size of microspheres increased by increasing the chitosan/acetic acid solution ratio and with decrease in stirring speed.

Encapsulation efficiency

The encapsulation efficiency of DCLD 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 (F10) was 64.40±0.04%. The encapsulation efficiency for F2-F12 was given in Table 3.

Percentage yield

% yield is more for microspheres prepared using homogenizer compared with microspheres prepared with mechanical stirrer as the particles are individual and not sticking to beakers and filter paper. The % yield for optimized formulation was (F10) 91.0. The percentage (%) yields for F2-F12 were given in Table 3.

FT-IR spectroscopy

FT-IR spectra of DCLD, chitosan, and DCLD loaded microspheres were compared. In DCLD spectrum (Fig. 2), the peak at 2963.4 cm^{-1} was related to Alkyl C-H stretch. The same peak was observed in optimized formulation (Fig. 3) at 2924.2c cm^{-1}. FT-IR spectra of DCLD the peak at 1726.8 cm^{-1} were related to aromatic C=C bending vibration. The same peak was observed in optimized formulation spectra at 1739 cm^{-1}.

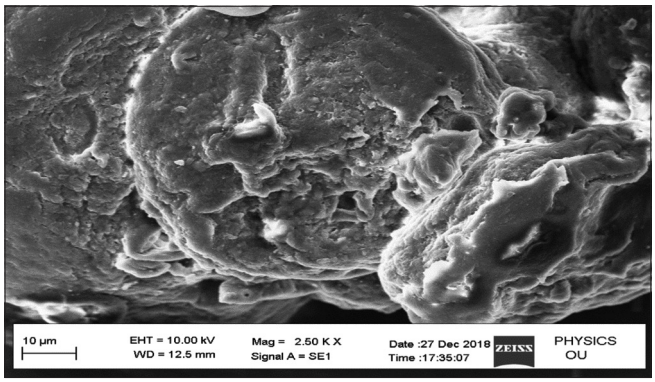

Fig. 1: SEM image of DCLD microspheres (F-10)

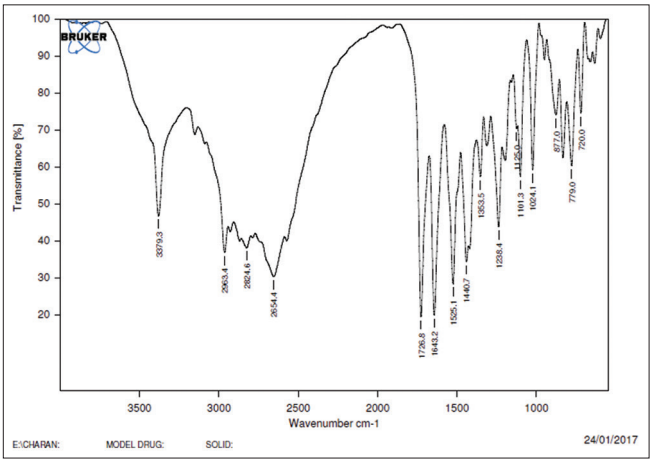

Fig. 2: FTIR spectrum of DCLD

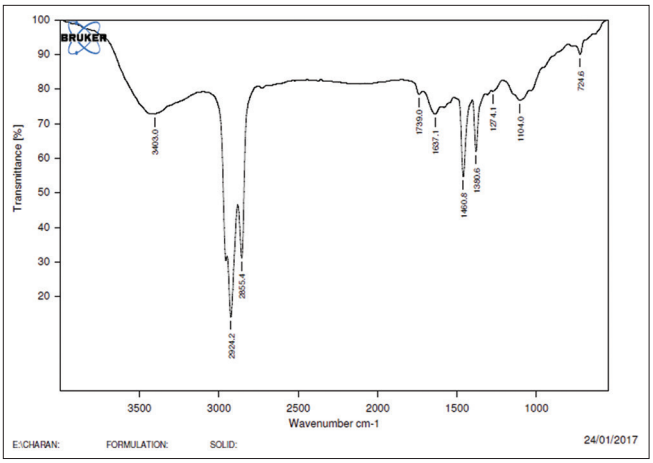

Fig. 3: FTIR of formulation (F-10)

The spectrum of chitosan (Fig. 4) showed peak at 1642.5 due to amine group. The spectrum of optimized formulation peak has shown at 1637 cm^{-1}, 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 revealed that the drug and polymer are compactable with each other.

Ex vivo permeation studies

The *ex vivo* permeation studies using goat intestinal membrane for formulations F2-F7 are given in Table 4 and Fig. 5, for formulations F8-F12 are given in Table 5 and Fig. 6, respectively. The amount of drug

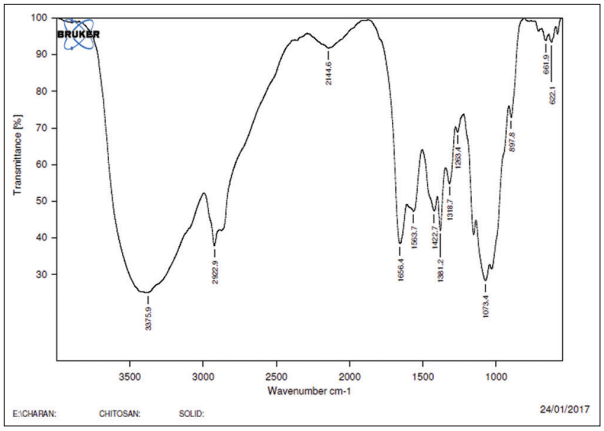

Fig. 4: FTIR of chitosan

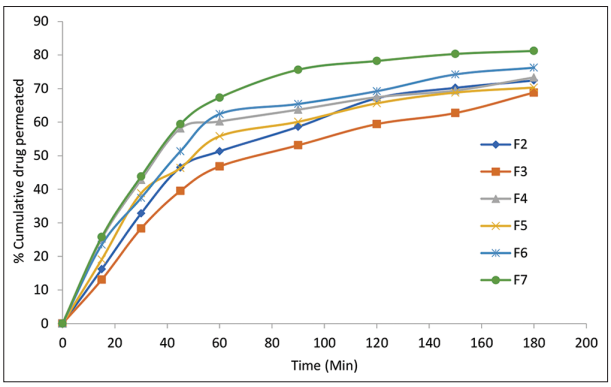

Fig. 5: *Ex vivo* permeation studies of formulations F2-F7

Table 2: Average particle size for formulations

Formulation code	Average particle size (µm) (n=3 ± SD)
F2	87.3±0.05
F3	98.5±0.80
F4	72.8±0.60
F5	83.1±0.08
F6	48.0±0.20
F7	39.2±0.90
F8	28.5±0.07
F9	23.3±0.60
F10	15.7±0.10
F11	13.3±0.09
F12	11.5±0.50

Table 3: Encapsulation efficiency and percentage (%) yield for formulations

Formulation code	% Encapsulation efficiency	% Yield
F2	47.8±0.05	53.2
F3	52.6±0.70	49.8
F4	63.0±0.40	58.0
F5	69.2±0.30	61.6
F6	58.6±0.05	73.2
F7	60.3±0.20	78.0
F8	56.7±0.60	70.0
F9	59.2±0.40	85.6
F10	64.4±0.30	91.0
F11	55.2±0.50	81.0
F12	50.5±0.20	89.0

permeated through goat intestine from different formulations depends on the concentration of polymer. The rate of permeation of drug from

Table 4: *Ex vivo* permeation studies of formulations F2-F7

Time (Min)/formulation code	% Cumulative drug permeated (n=3 ± SD)					
	F2	F3	F4	F5	F6	F7
0	0.0	0.0	0.0	0.0	0.0	0.0
15	16.2±0.7	13.1±0.3	25.3±0.8	19.1±1.5	23.5±0.8	25.8±0.6
30	32.8±2.1	28.3±1.7	42.8±1.9	38.8±0.9	37.4±2.0	43.8±1.3
45	46.5±2.3	39.5±0.3	58.1±0.2	46.3±0.4	51.3±0.4	59.4±0.5
60	51.3±1.6	46.8±1.1	60.2±0.3	55.8±1.2	62.4±0.3	67.3±0.4
90	58.6±0.4	53.1±0.5	63.7±0.5	60.1±0.8	65.4±0.6	75.6±0.7
120	67.1±0.9	59.4±0.4	67.4±0.7	65.6±0.3	69.2±0.4	78.2±0.3
150	70.2±0.7	62.7±0.5	69.4±1.5	68.8±0.6	74.2±0.3	80.3±0.6
180	72.4±0.4	68.8±0.3	73.3±0.2	70.3±0.4	76.2±1.0	81.2±0.5

Table 5: *Ex vivo* permeation studies of formulations F8-F12 and pure drug

Time (min)/formulation code	% Cumulative drug permeated (n=3 ± SD)					
	F8	F9	F10	F11	F12	Pure drug
0	0.0	0.0	0.0	0.0	0.0	0.0
15	28.4±0.6	26.1±2.4	32.2±1.8	35.3±1.3	37.3±0.6	35.0±0.2
30	47.4±0.5	48.2±0.5	54.4±0.4	51.6±0.6	50.6±1.3	38.0±0.7
45	60.2±1.4	63.5±0.4	71.6±0.7	66.7±0.4	68.8±0.4	43.0±0.6
60	72.6±0.3	75.2±0.3	83.3±0.6	78.4±0.8	75.6±0.8	49.0±0.8
90	78.4±0.2	80.1±0.8	87.6±0.3	85.0±1.0	80.4±0.6	61.0±0.4
120	82.3±0.4	84.4±1.5	94.4±1.2	91.1±1.1	89.3±1.5	67.0±1.5
150	83.1±0.4	87.5±0.5	97.5±0.5	94.1±0.2	90.3±0.2	72.0±0.4
180	85.6±0.8	90.1±0.2	97.7±0.6	94.2±0.1	92.4±0.3	78.0±0.2

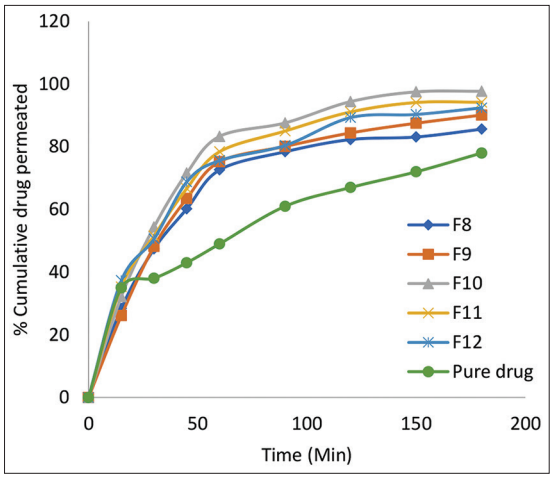

Fig. 6: *Ex vivo* permeation studies of formulations F8-F12 and pure drug

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, 83.3% of drug was permeated with in 60 min whereas for pure drug 49% of drug was permeated with in 60 min.

CONCLUSION

DCLD microspheres were successfully developed using chitosan to glacial acetic acid ratio 10 by emulsion cross linking technique with process parameter 1500 rpm with homogenizer. The optimized formulation (F10) shown *ex vivo* permeation of 83.3% within 60 min and particle size of 15.70±0.05 µm. The drug diffusion from optimized formulation (F10) was good compared to pure drug. This enhanced permeability will improve the absorption.

The previous reports pointed out that the microspheres with the size range of 5–25 µm have notable liver targeting [8]. The optimized formulation has average particle size of 15.70±0.05 µm; hence, it may helpful to accumulate the more amount of drug in liver. Further *in-vivo* studies should be carried out using animal models to investigate the drug accumulation in the liver.

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AUTHORS CONTRIBUTIONS

Jeevana Jyothi B has designed the plan of present work and responsible for this novel work and preparation of manuscript. K.Sudhamanihas performed the experiments involved in the present research work.

CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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