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Development and evaluation of polybutylcyanoacrylate microcapsules of daclatasvir dihydrochloride

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Abstract
Daclatasvir dihydrochloride (DCLD) is useful for treatment of hepatitis caused by Hepatitis C virus. The present work is aimed at development of polybutylcyanoacrylate microcapsules and to evaluate at *In-vitro* level including pharmacokinetic parameters using albino rats. PBCA microcapsules of DCLD were prepared by emulsion polymerization method. Eight formulations like F1 and F8 were tried. Six formulations such as F3 to F8 were obtained in discrete microcapsule form. Their particle size ranged in 126.3 to 499.7 µm was satisfactory. Percent drug content values in the range of 96.5% ±0.89 to 99.7%±0.56. *Ex-vivo* permeation studies revealed 99.3% of drug diffusion from formulation F5 after 24 hrs indicating fair GI absorption. Drug diffusion from pure drug was also reasonable with 51.9 %. But pharmacokinetic parameters are further estimated for formulation F5 to assess elimination half life of F5 compared to pure drug as this study will give an idea about prolonged therapeutic effect of the drug against HCV virus. There is enhancement of $t_{1/2}$ (3.68 hr), AUC_{0-12}(3960ng.hr/ml), and K_a (0.98hr-1) of PBCA microcapsules compared to pure drug indicating enhanced bioavailability of drug which will give way for prolonged exposure of virus present in blood to the drug to treat hepatitis C virus.

Keywords: daclatasvir dihydrochloride, hepatitis C virus, polybutylcyanoacrylate microcapsules, emulsion polymerization, *Ex-vivo* permeation studies, enhanced bioavailability

Introduction
Hepatitis [1], is the inflammation of liver cells which can progress to fibrosis (scarring), cirrhosis or liver cancer. Five main types of hepatitis are caused by viruses and usually Hepatitis C is commonly spread via direct contact with the blood of a person who has the disease.

Hepatitis C virus (HCV) causes both acute and chronic infection. Acute HCV infection is usually asymptomatic, and is only very rarely associated with life-threatening disease. About 15–45% of infected persons spontaneously clear the virus within 6 months of infection without any treatment. The remaining 55–85% of persons will develop chronic HCV infection. Of those with chronic HCV infection, the risk of cirrhosis of the liver is 15–30% within 20 years [2, 3]. Treatment involves lifestyle changes to help prevent further damage in liver and reduce the risk of spreading the infection. Use of combination of two or three medications to fight the virus is known as combination therapy. The medication used for 12 to 48 weeks. Two main medicines such as pegylated interferon and ribavirin are in use. These medications were frequently taken together, but nowadays they are often combined with a third medication, such as simeprevir or sofosbuvir. At this context, FDA has approved another molecule such as DCLD which belong to newer hepatitis C medications that have been shown to make treatment more effective [4]. DCLD acts by reducing RNA of hepatitis C virus rapidly within first 6hrs of dosing [5]. It attaches selectively NS5A polymerase and stops further replication [6].

There are no reported formulations of DCLD and hence present research was aimed for preparation of polybutylcyanoacrylate microcapsules [7, 8] and to evaluate at *In-vitro* level including assessment of the pharmacokinetic parameters such as $C_{max,}$, $t_{max,}$, $AUC_{0-24,}$ $t_{1/2,}$ K_a and K_e using albino rats.

Materials and Methods
Daclatasvir dihydrochloride was obtained as gift sample from Mylan labs., Hyderabad, Polybutylcyanoacrylate was purchased from Sungrace Scientific laboratories., Mumbai. Chloroform, ethanol, span 85,cyclohexane, polysorbate 20, methanol HPLC grade, acetone were purchased from Hi media Ltd.,, Water HPLC was purchased from Molychem, Mumbai and all other chemicals are of analytical grade.

Preparation of PBCA microcapsules of Daclatasvir dihydrochloride [9, 10, 11]
PBCA microcapsules of daclatasvir dihydrochloride were prepared by emulsion polymerization method due to good water solubility of DCLD. Trial formulations F1 to F8 (8 no.) were prepared by changing various formulation ingredients as shown in Table 1. Aqueous phase was prepared by dissolving DCLD in distilled water. This was added with stirring to half of the total volume of organic phase [chloroform: cyclohexane: 1: 4**]** containing 5%V/V Span 85. Poly butylcyanoacrylate monomer was added to remaining half of organic phase and was added to above solution. Stirring was continued for 30 min. Then cyclohexane was added to quench the reaction and to reduce possibility of polymerization occurring between the formed microcapsules. The microcapsules were sediment in few minutes. Then organic phase was evaporated. Further polysorbate-20 and ethanol were added and the suspension was stirred well. When the

microcapsules are sedimented the solution was centrifuged for 1 hr. Then the supernatent liquid was decanted, the product was air dried and packed.

Table 1: Composition Of Daclatasvir Dihydrochloride Microcapsules.

Ingredient	Formulations							
	F1	F2	F3	F4	F5	F6	F7	F8
DCLD (mg)	60	120	30	60	120	180	240	300
Cyclohexane (ml)	8	8	16	16	16	32	32	40
Chloroform (ml)	2	2	4	4	4	8	8	10
Polybutylcyano acrylate (ml)	0.25	0.25	0.20	0.25	0.30	0.35	0.40	0.45
Span85	0.5	0.5	0.5	0.5	0.5	1.0	1.0	1.5
Polysorbate	-	-	10	10	10	20	20	30
Ethanol (ml)	-	-	20	20	20	30	30	40

Evaluation of daclatasvir dihydrochloride microcapsules

Microcapsules were obtained in case of trials F3 to F8 and failed in case of F1 and F2. Formulations F3 to F8 were evaluated by estimation of percent drug content, particle size, phase contrast microscopy, FTIR, *ex-vivo* diffusion studies and ***in-vivo*** pharmacokinetic parameters.

Estimation of drug content

Microcapsules equivalent of 60 mg of DCLD were crushed in a glass mortar and suspended in 100 ml of phosphate buffer pH 7.4. Then keep aside for 24 hours. After 24 hr, the solution was filtered and 1 ml samples were diluted to 10 ml and analyzed for the drug content using UV Visible spectrophotometer (Shimadzu-1700) at 214 nm.

Particle size determination

The particle size determination of drug loaded microcapsules, F3 to F8 was carried out using optical microscopy along with a stage micrometer having an accuracy of 0.01 mm. The eye piece micrometer was calibrated by using a standard stage micrometer at 45X (high power). A suspension of microcapsules in liquid paraffin was prepared in a beaker and then one drop of this was dropped on a clean glass slide and covered with a cover slip. The average sizes of 50 microcapsules were determined for each formulation using the calibration factor. The average particle size of the microcapsules was determined by using Edmondson's equation. Dmean= Σnd / Σn Where, n = Number of microcapsules checked; d = Mean size range.

Ex-*vivo* Diffusion studies [12]

Ex-vivo absorption studies were carried out for pure drug and formulations F3 to F8 (equivalent to 60 mg of drug). Modified Franz diffusion cell with a receiver compartment of 25 ml volume and effective diffusion area of 2.5 cm^2 was used for the study. Fresh intestinal membrane of goat was collected from slaughter house and used in absorption experiments. The receptor compartment was filled with 25 ml of pH 7.4 phosphate buffer maintained at 37 ± 0.5^0C and stirred by a magnetic bar at 100 rpm. 60 mg of pure DCLD and microcapsule formulations (F3 to F8) was placed on membrane by using Franz diffusion cell apparatus. The top of the cell was covered. At appropriate time intervals 3 ml aliquots of the receptor medium were withdrawn and immediately replaced by an equal volume of buffer to maintain sink conditions. Correction factors for each aliquot were considered in calculation of release profile. They were analyzed for the drug content by UV-visible spectrophotometer λ_{max} of 214 nm.

Phase contrast microscopy

Small amount of microcapsule of F5 was placed on a glass slide and spread over. Then the microscopy was adjusted to 10X and focused by adjusting the stage micrometer. Then the shape of the microcapsules was observed and the photograph was taken.

FTIR analysis [13]

FTIR spectra of pure DCLD and drug loaded microcapsules (F5) was obtained on a FTIR Spectrophotometer (Bruker, JAPAN) equipped with a DTSG detector. Samples were prepared by KBr pressed pellet technique. The scanning range was 400 to 4000^{-1} and the resolution was $4cm^{-1}$.

Pharmacokinetic Studies

Experimental Design

In--vivo studies were conducted for promising microcapsules of DCLD, F5 in comparison with the respective pure drug DCLD using male wistar albino rats weighing 180-200 gm. The studies were approved by Animals ethical committee, Sri Padmavati Mahila Visvavidyalayam (1677/PO/Re/S/2012/ CPCSEA/19 Dtd., 06/05/2016). Powder equivalent of 9.2 mg/Kg of rat weight of DCLD was administered to rats. Rats were divided in to 3 groups each consisting of six animals. Group I was treated with control, Group II with pure drug and Group III with F5.

Sample collection, processing and estimation of DCLD

At predetermined time intervals, blood samples (0.2-0.3 ml) were collected from retro orbital plexus of rats into eppendrof tubes containing dipotassium ethylene diamine tetra acetic acid. The collected blood samples were coagulated by centrifugation at 3000 rpm for 20 min and plasma was separated and stored in deep freezer until used. Plasma samples of DCLD were thawed after withdrawal from deep freezer. The plasma concentration of DCLD was estimated by HPLC method (Shimadzu, Sphinchrome software series, Japan; Luna C_{18} (250 mm x 4.6 mm, 5 µm) column, Detection

at 224 nm using SPDMP-10A photo diode array detector. methanol and water 80:20 v/v as mobile phase).

Plasma concentration of drugn following the administration of pure drug and microcapsule formulation (F5) was calculated from the peak area ratios of drug and internal standard and bioavailability curves are drawn between plasma conc. Vs. time**.** Various pharmacokinetic parameters such as peak plasma concentration (C_{max}), time at which C_{max} occurred (T_{max}), area under the curve (AUC), elimination rate constant (K_e), biological half-life ($t_{½}$), absorption rate constant (K_a) and relative bioavailability were calculated by using method of residuals.

Results and Discussion

Among 8 trial formulations six formulations F3 to F8 were obtained in discrete microcapsule form and were evaluated to select promising formulation. Percent drug content values obtained in the range of 96.5% ±0.89 to 99.7%±0.56 and were found satisfactory (Table 2). Particle sizes in the range of 126.3 to 499.7µm was also satisfactory. Particle size values indicates that as polymer concentration is increased from F3 to F5 the particle size is also increased. Reduction in stirring speed from F6 to F8 reduced particle size.

Table 2: particle size & percentage drug content pbca microcapsules of dcld

Formulations	Particle size(µm)	% drug content
F3	126.3	96.5±0.61
F4	254.6	97.6±0.32
F5	499.7	99.7±0.55
F6	407.1	98.9±0.68
F7	326.2	98.5±0.65
F8	164.5	97.2±0.54

The results of *ex-vivo* permeation studies of DCLD microcapsules are shown in Table 3. Drug diffusion was good from prepared formulatons F3 to F5 compared to pure drug. F5 evidenced 99.3% after 24 hrs indicating fair GI absorption of DCLD microcapsules from intestinal membrane. Drug diffusion from pure drug also is reasonable in such a way that there is 51.9 % release in first one hour. Hence the pharmacokinetic parameters are further estimated for formulation F5 to assess enhancement of elimination half life of F5 compared to pure drug. This assessment will give an idea about prolonged stay of the drug in the blood circulation to act against HCV virus in blood which is responsible for liver cirrhorrosis.

Table 3: % drug diffused from pure drug and microcapsules f3 to f8

Time (hrs)	Percent drug released						
	Pure	F3	F4	F5	F6	F7	F8
0.25	70.2±0.41	12.6±0.61	20.1±0.61	30.5±0.31	28.01±0.21	24.3±0.11	16.3±0.31
0.5	72.5±0.22	15.5±0.32	24.3±0.32	34.3±0.53	31.6±0.53	27.4±0.56	19.6±0.25
0.75	75.6±0.55	21.3±0.65	30.4±0.12	42.5±0.21	39.3±0.12	33.4±0.34	25.3±0.51
1	77.3±0.48	28.4±0.68	35.6±0.65	51.9±0.64	43.1±0.85	39.6±0.28	31.5±0.34
1.5	79.9±0.36	32.3±0.45	40.3±0.68	58.6±0.86	49.6±0.62	42.4±0.49	36.7±0.28
2	81.5±0.65	39.1±0.59	49.2±0.45	61.3±0.71	56.3±0.41	51.3±0.37	42.5±0.16
3	83.2±0.54	44.6±0.1	58.6±0.59	64.1±0.52	61.6±0.20	60.01±0.26	51.7±0.24
4	84.4±0.13	52.7±0.42	64.01±0.1	72.2±0.21	70.3±0.43	69.6±0.54	59.01±0.35
5	86.7±0.42	60.2±0.52	70.2±0.10	80.4±0.32	79.6±0.59	76.2±0.36	65.3±0.49
6	88.3±0.62	69.6±0.32	80.3±0.52	88.1±0.13	84.3±0.61	82.6±0.43	78.5±0.52
12	93.8±0.22	73.5±0.18	84.4±0.32	94.6±0.31	89.3±0.37	85.3±0.22	79.6±0.41
24	96.6±0.28	77.8±0.54	86.6±0.18	99.3±0.41	92.3±0.54	89.6±0.14	83.3±0.39

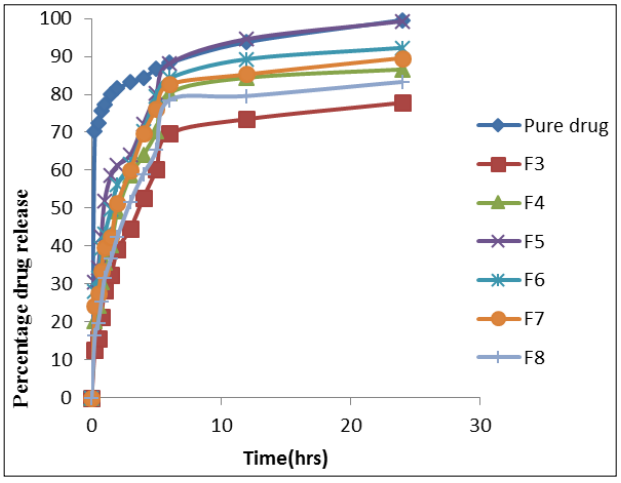


Fig 1: percentage drug diffused from pure dcld and prepared dcld microcapsules (f3 to f8) vs. Time

Surface morphology of promising formulation F5 is depicted in Fig.2. The microcapsules are discrete and spherical with rough outer surface morphology which could be because of the surface association of the drug with the polymer.

Fig 2: Phase contrast microscopy photograph of microcapsules of DCLD, F5

FT-IR spectra of pure DCLD and drug loaded microcapsules F5 are shown in Fig.4 and 5. The characteristic peaks of pure drug were observed due to aromatic C-H bending at 947.42cm^{-1}, amide C=O stretch at 1634.89cm^{-1}, aromatic C=C bending at 1733.38cm^{-1} and alkyl C-H stretch at 2543.31cm^{-1}.

Then the characteristic peaks of microcapsule formulation F5 was C-H bending at 935.02cm^{-1}, amide C=O stretch at 1637.46cm^{-1}, aromatic C=C bending at 1749.67 cm^{-1} and alkyl C-H stretch at 2858.31cm^{-1}. The FT-IR spectrum of drug loaded microcapsules also revealed all characteristic peaks of the pure drug with shift of peaks. Hence it is concluded that there is no interaction between the drug and polymers.

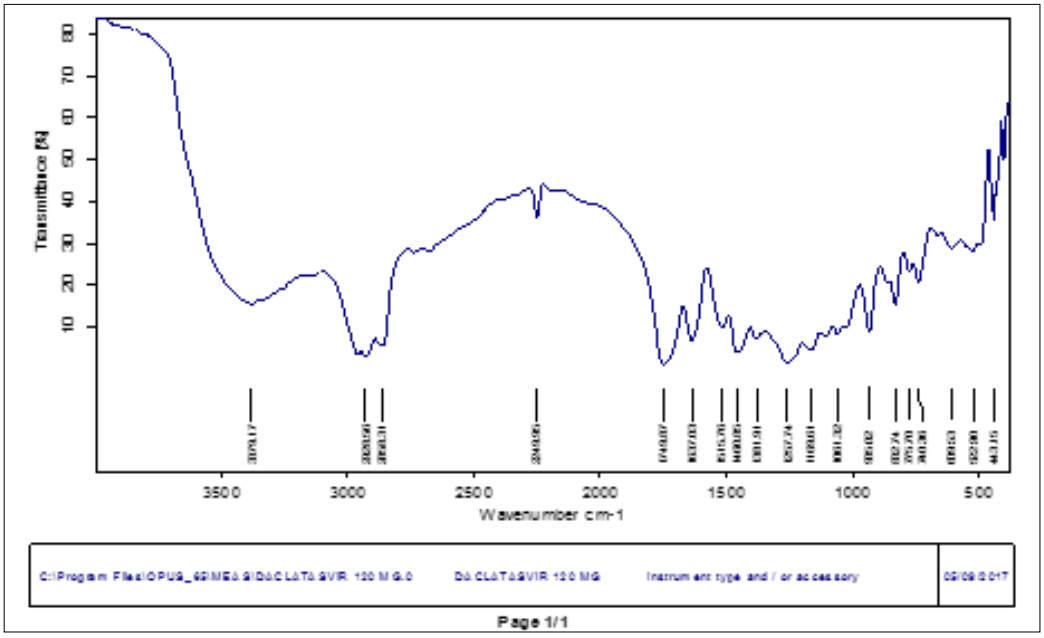


Fig 4: FTIR spectrum of pure DCLD

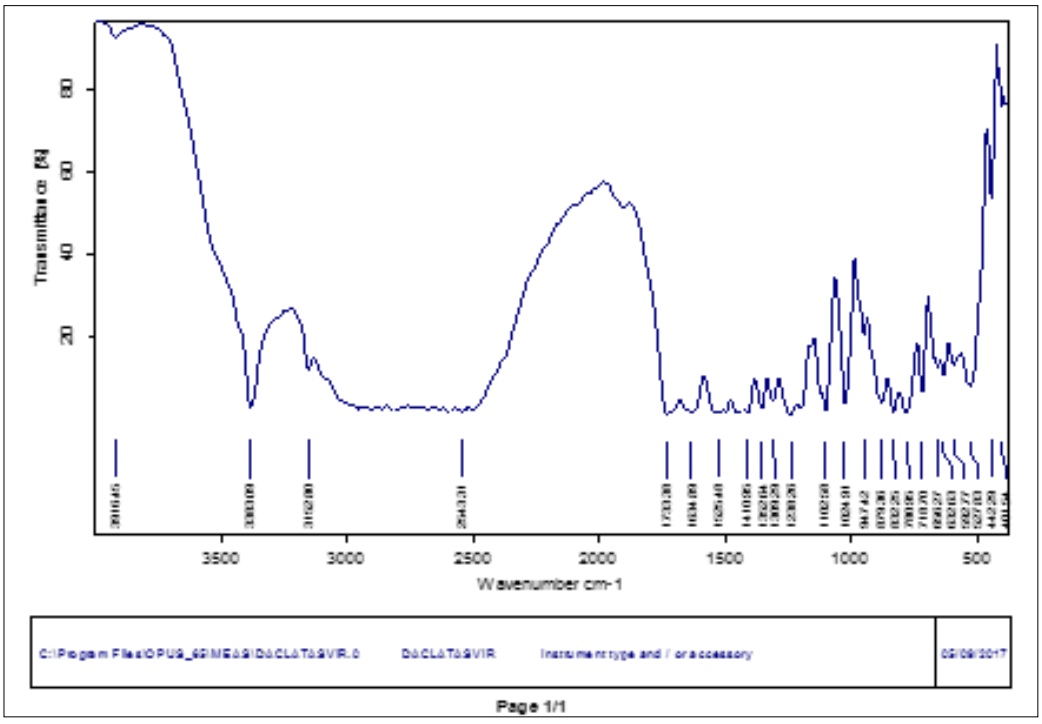


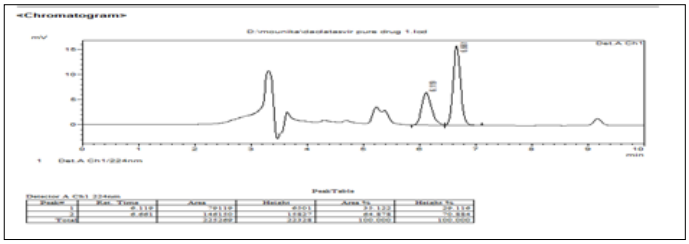
Fig 5: FTIR Spectrum of Microcapsule Formulation of Dcld, F5

Pharmacokinetic Studies

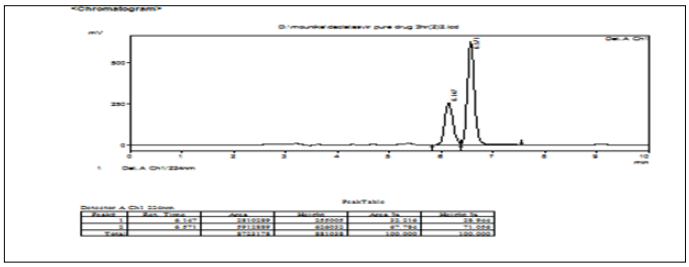
DCLD concentration in plasma following the administration of pure drug and microcapsule formulation (F5) was calculated from the peak area ratios of drug and internal standard.

The relevant chromatograms of pure drug and microcapsules, F5 are shown in Fig. 6 and Fig.7. Bioavailability curves are drawn between plasma conc. vs. time as shown in Fig 8.

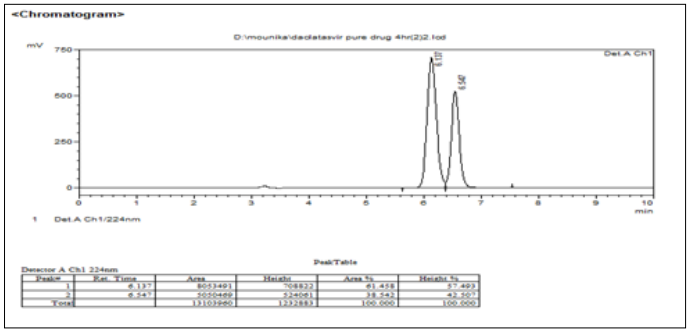
After 0.5 hour



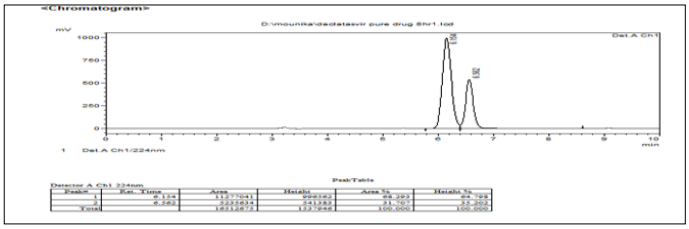
1.0 hours



4 hours



8 hours



12 hrs

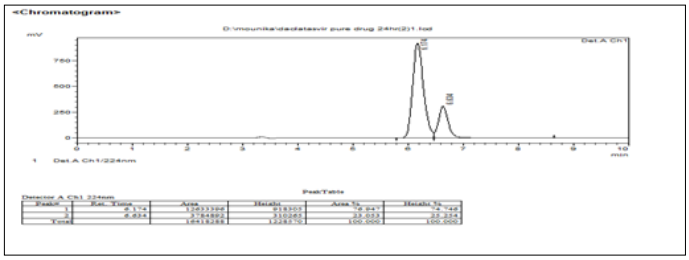
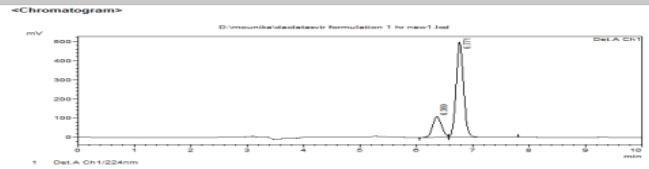


Fig 6: HPLC chromatogram after administration of pure drug, dcld spiked with i.s at various time intervals

after 0.5 hour

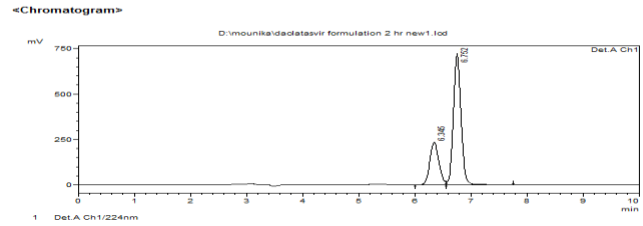


PeakTable

Detector A Ch1 224nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	6.389	1803564	358742	20.823	17.661
2	6.777	4733113	466672	79.168	82.339
Total		5781122	80441?	100.000	100.000

1.0 hours

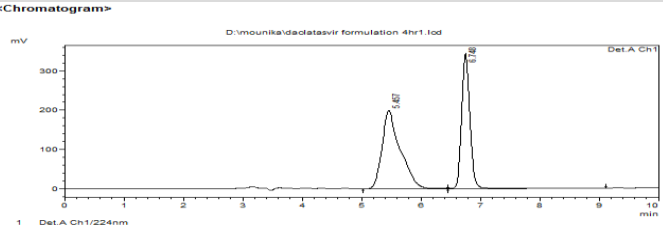


PeakTable

Detector A Ch1 224nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	6.345	2630586	233898	27.868	24.460
2	6.752	6808706	722341	72.132	75.540
Total		9439292	956239	100.000	100.000

4 hours

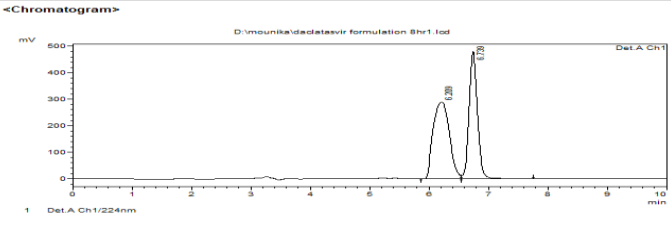


PeakTable

Detector A Ch1 224nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	5.457	4229033	199187	55.839	36.628
2	6.748	3368245	344629	44.161	63.372
Total		7627280	543816	100.000	100.000

8 hours

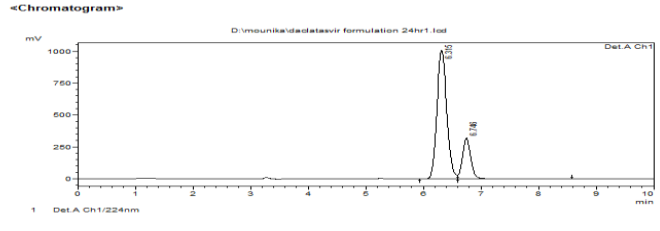


PeakTable

Detector A Ch1 224nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	6.209	5506707	289542	53.886	37.629
2	6.739	4712391	479922	46.114	62.371
Total		10219098	769463	100.000	100.000

12 hrs



PeakTable

Detector A Ch1 224nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	6.315	11919252	1008449	78.584	75.807
2	6.746	3248234	321840	21.416	24.193
Total		15167486	1330289	100.000	100.000

Fig 7: HPLC chromatogram after administration of microcapsules, F5 spiked with i.s at various time intervals

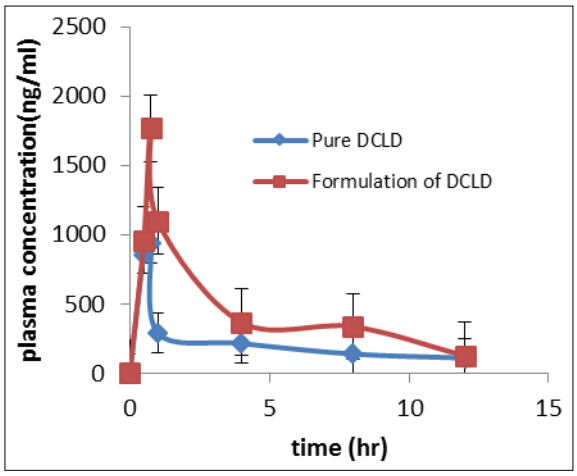


Fig 8: plasma concentration vs. Time curves of pure dcld and microcapsules of dcld, F5

Pharmacokinetic parameters that are calculated for pure drug and microcapsules of DCLD are shown in Table 5.

Table 4: pharmacokinetic parameters values of pure drug and microcapsules, F5.

S.no	Pharmacokinetic parameters	Pure drug (DCLD)	Microcapsule formulation (F5)
1	K_a	0.89 hr^{-1}	0.98 hr^{-1}
2	K_e	0.20 hr^{-1}	0.16 hr^{-1}
3.	AUC	2823 ng.hr/ml	3960 ng.hr/ml
4.	Volume of distribution (V_d)	3.6ltrs	2.2ltrs
5.	c_{max}	850ng/ml	990 ng/ml
6.	t_{max}	2.1 hr	3.2 hr
7.	$t_{1/2}$	3.46 hr	3.68 hr

The values shows that, there is enhancement of $t_{1/2}$ (3.68 hr), AUC_{0-12} (3960ng.hr/ml), and K_a (0.98hr-1) of PBCA microcapsules of daclatasvir dihydrochloride compared to pure drug parameters $t_{1/2}$ (3.46 hr), AUC_{0-12} (2823ng.hr/ml), and K_a (0.89hr-1). Reasonable enhancement of AUC_{0-12} from 2823 ng.hr/ml to 3960 ng.hr/ml and half-life indicated enhanced bioavailability of drug which will give way for prolonged exposure of virus present in blood to the drug. Hence it is concluded that microcapsule formulation of daclatasvir dihydrochloride is a promising approach to treat hepatitis C virus**.**

Conclusion

Poly butyl cyanoacrylate microcapsules of daclatasvir dihydrochloride is a promising approach to treat hepatitis C virus with enchanced C_{max}, $t_{1/2}$, reduced K_e and hence enhanced presence of drug in blood for effective treatment of hepatitis C virus. This can be used along with Interferon with better results to treat hepatitis C.

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