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Optimization and Characterization of Solid Lipid Nanoparticles for Treatment of Hepatitis

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Abstract

Hepatitis B virus (HBV) infection continues to remain a significant global health problem. Estimates of the World Health Organization (WHO) suggest that more than 2 billion people worldwide have been infected with HBV. Of these, approximately 240 million individuals have chronic (long-term) liver infections and at risk of serious illness and death, mainly from liver cirrhosis and hepatocellular carcinoma (HCC). More than 780000 people die every year due to the acute or chronic consequences of hepatitis B.

In present research work, Telbivudine loaded solid lipid nanoparticles were prepared by emulsification- Sonification method with slight modification. The Optimization was done by design of expert (Design Expert ™ Software). Total 36 batches were prepared by using different types of lipids, type of surfactant and concentration of surfactant and it was optimized on basis of entrapment efficiency and particle size. The optimized formulation was evaluated for Zeta potential and particle morphology by SEM and further it is modified by galactose for active targeting (GmSLN)

The GmSLN was further evaluated by In-Vitro Hepatoprotective activity and ex vivo cellular uptake studies. Hepatoprotective activity was performed against DCFH-hepatotoxicity using HepG2 Liver cell lines. The Cellular Uptake Studies was performed using Hepa-1c1c7 cells. The results of Hepatoprotective activity against DCFH-hepatotoxicity showed that DCFH-toxicated cells were recovered to about 67% and 86% upon treatment with 200 µg/ml of SLN and GA modified SLN, respectively. The time dependent cellular uptake is also studied which reveals that GA- modified system shows better uptake in less time as compared to Plain SLN which proves that modification increases uptake in hepatocyte cells after modification with galactose.

1. INTRODUCTION:

Five hepatitis viruses are the leading causes of liver disease worldwide. Among them, hepatitis B virus (HBV) is responsible for approximately 350 million chronic infections worldwide and over one million annual deaths.

Hepatitis B virus (HBV) infection continues to remain a significant global health problem. Estimates of the World Health Organization (WHO) suggest that more than 2 billion people worldwide have been infected with HBV. Of these, approximately 240 million individuals have chronic (long-term) liver infections and at risk of serious illness and death, mainly from liver cirrhosis and hepatocellular carcinoma (HCC). More than 780000 people die every year due to the acute or chronic consequences of hepatitis B.

Since India has one-fifth of the world's population, it accounts for a large proportion of the worldwide HBV burden. India harbors 10–15% of the entire pool of HBV carriers of the world. It has been estimated that India has around 40 million HBV carriers. About 15–25% of HBsAg carriers are likely to suffer from cirrhosis and liver cancer and may die prematurely.

Lipid nanoparticles have many advantages in comparison to other particulate systems such as: -

- The ease of large-scale production.
- Biocompatible and biodegradable nature of the materials.
- low toxicity potential.
- Possibility of controlled and modified drug release.
- Drug solubility enhancement and the possibility of both hydrophilic and lipophilic drug incorporation.

The main objective of study is to prepare and optimize SLNs containing anti-viral drug for treatment of hepatitis B. The SLNs was attached with targeting moieties to target specific receptors on hepatocytes cells for treatment of hepatitis B.

2. EXPERIMENTAL WORK

2.1 Preparation of Solid Lipid Nanoparticles (SLN)

The Telbivudine loaded solid lipid nanoparticles were prepared by emulsification- Sonification method with slight modification. Briefly, required amount of lipid were dissolved in a 10 mL mixture of Chloroform and Methanol (1:1 v/v) at 70 °C. The melt was slowly poured into aqueous phase maintained at the same temperature containing Telbivudine. The Surfactant was added in either lipid phase or aqueous phase, depending on its type. The Mixture was stirred by Homogenizer at 4000 rpm for 30 min. This suspension was then sonicated using probe sonicator for 5 min. The Cycle of Probe Sonicator is 5 Sec running and 2 sec stop and maximum temperature set was 400C so that lipid may not get degrade during sonication. The resulting dispersion was filtered through membrane filter (0.45 µm) to remove any excess lipid and obtained SLN suspension.

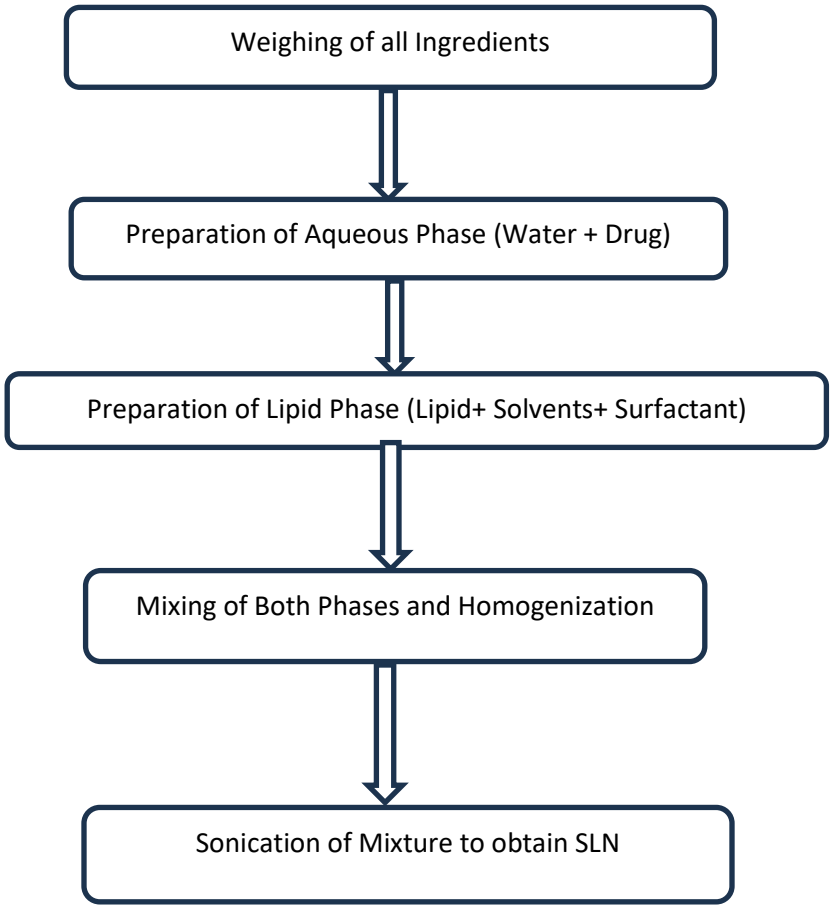


Fig no 1: Steps for preparation of Solid lipid nanoparticles

2.2 Optimization of Solid Lipid Nanoparticles

The Optimization was done by design of expert (Design Expert TM Software). Total 36 batches were prepared by using different types of lipids, type of surfactant and concentration of surfactant and it is optimized on basis of entrapment efficiency and particle size. The different batches were prepared using 10 % and 15% lipid Concentration

Table 1: Formulation of different Batches with Lipid Concentration (10%)

Formulation Code	Lipid	Surfactant
SLN-1	Stearic Acid	Span 20 (1 %)
SLN-2	Stearic Acid	Span 20 (2 %)
SLN-3	Stearic Acid	Span 20 (3 %)
SLN-4	GMO	Span 20 (1 %)
SLN-5	GMO	Span 20 (2 %)
SLN-6	GMO	Span 20 (3 %)
SLN-7	GMS	Span 20 (1 %)
SLN-8	GMS	Span 20 (2 %)
SLN-9	GMS	Span 20 (3 %)
SLN-10	Stearic Acid	Tween 80(1 %)
SLN-11	Stearic Acid	Tween 80 (2 %)
SLN- 12	Stearic Acid	Tween 80 (3 %)
SLN- 13	GMO	Tween 80(1 %)
SLN- 14	GMO	Tween 80 (2 %)
SLN- 15	GMO	Tween 80 (3 %)
SLN- 16	GMS	Tween 80(1 %)
SLN- 17	GMS	Tween 80 (2 %)
SLN- 18	GMS	Tween 80 (3 %)

Table 2 : Formulation of different Batches with Lipid Concentration (15%)

Formulation Code	Lipid	Surfactant
SLN-19	Stearic Acid	Span 20 (1 %)

SLN-20	Stearic Acid	Span 20 (2 %)
SLN-21	Stearic Acid	Span 20 (3 %)
SLN-22	GMO	Span 20 (1 %)
SLN-23	GMO	Span 20 (2 %)
SLN-24	GMO	Span 20 (3 %)
SLN-25	GMS	Span 20 (1 %)
SLN-26	GMS	Span 20 (2 %)
SLN-27	GMS	Span 20 (3 %)
SLN-28	Stearic Acid	Tween 80(1 %)
SLN-29	Stearic Acid	Tween 80 (2 %)
SLN- 30	Stearic Acid	Tween 80 (3 %)
SLN- 31	GMO	Tween 80(1 %)
SLN- 32	GMO	Tween 80 (2 %)
SLN- 33	GMO	Tween 80 (3 %)
SLN- 34	GMS	Tween 80(1 %)
SLN- 35	GMS	Tween 80 (2 %)
SLN- 36	GMS	Tween 80 (3 %)

2.3 Characterization of Optimized Formulation

2.3.1. Zeta Potential

The Zeta Potential of Solid Lipid Nanoparticles were determined using photon correlation spectroscopy (PCS) (Zetasizer, HAS 3000; Malvern Instruments, Malvern, UK) from RGPV bhopal. The analysis was performed at a fixed angle of 90◦ at 250C using sample appropriately diluted with filtered water (0.2 µm filter, Minisart, Gottrgen, Germany).

2.3.2. Particle Morphology

Morphologic evaluation of the solid lipid nanoparticles was performed using scanning electron microscope (SEM; Stereoscan 440, lecia). For SEM analysis solid lipid nanoparticles were placed on the glass slide and

coated with gold prior to examination by SEM. The voltage was ranged from 10 to 25kV during scanning. The SEM analysis was done from Punjab University Chandigarh and Averin Biotech Hyderabad.

2.4 Preparation of Galactose modified SLN (GmSLN)

Firstly d-galactose (2 mg/ml) was dissolved in 0.1 M sodium acetate buffer (pH 4.0) and 1 ml of Epichlorohydrin as linker was added and afterward it was added to uncoated SLNs (SLN-D) dispersion. The mixture was allowed to continuously be agitated on a magnetic stirrer (Remi, Mumbai, India) maintained at an ambient temperature for 24 hours to make sure the completion of reaction. GA-modified SLN (GmSLN) were subjected to extensive dialysis against double distilled water (DDW) in a dialysis tube (dialysis bag; MWCO 12 kDa, Himedia, India) for 30 min to remove free galactose and other impurities. The GmSLN was confirmed by Fourier transform infrared (FTIR).

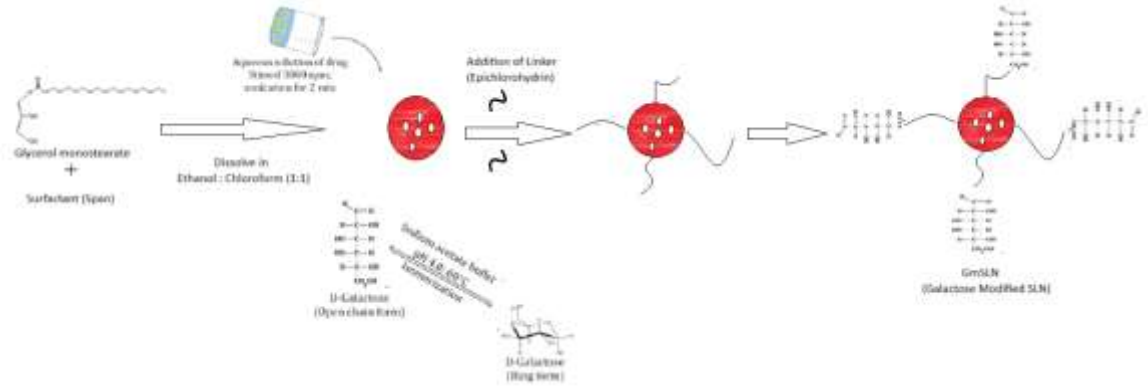


Figure no 2: Scheme for Modification of Galactose to SLN

2.5 *In-vitro* release studies

In-vitro release studies were performed using Franz diffusion cell with a surface area of 3.14cm2. The dialysis membrane was mounted between donor and receptor compartments of the diffusion cell with stratum corneum facing the donor compartment. Solid lipid nanoparticles were placed in the donor compartment and 15ml of phosphate buffer saline (PBS, pH 7.4) was placed in receptor compartment. The contents of receptor compartment were agitated over a magnetic stirrer. The study was conducted at 37◦C and samples of 1ml was collected at predetermined time points and replaced with PBS (pH 7.4). The cumulative amount of drug release was determined using UV spectrophotometer at 267 nm.

2.6 Hepatoprotective activity against DCFH-hepatotoxicity

DCFH (2,7-Dichlorofluorescein) is generally used to measure in vitro oxidative stress generated by free radicals through the principle of oxidation of DCFH to the fluorescent DCFH (Rota et al., 1999). In the present study, we used DCFH as an inducer of in-vitro hepatotoxicity. The approved nucleoside analog-based anti-HBV drug, lamivudine was used as standard. HepG2 cells were seeded in a 96-well flatbottom plate and grown overnight. The concentration of DCFH that caused 50% inhibition of cell proliferation (IC50: 100 µg/ml), was used as a cytotoxic dose (Al-Yahya et al., 2013; Arbab et al., 2015), prepared in DMSO. The culture monolayer was replenished with RPMI-1640 containing 100 µg/ml DCFH plus a dose of plant fraction (25, 50, 100 and 200 µg/ml), including untreated as well as DCFH only-treated controls. All samples were in triplicate. The treated cells were incubated for 48 h at 370 C followed by MTT assay. The optical density (OD) was recorded at 570 nm in a microplate reader (BioTek, ELx800) and the data analyzed.

2.7 Cellular Uptake Studies

The Cellular Uptake Studies is an important parameter to check the internalization of different prepared SLN and surface modified systems. Hepa-1c1c7 cells were seeded onto a glass-bottomed culture dish (MatTek Corp., Ashland, MA) and incubated with fresh a-MEM supplemented with 5% feral bovine serum (biowest, Nuaille´, France) for 24 h prior to treatment. The cells were treated with different drug loaded SLN in the medium. After the incubation, the cells were fixed with 4% paraformaldehyde and 0.2% picric acid in 100

mM sodium phosphate buffer (pH 7.2) overnight. All the test samples were prepared by adding FITC dye (100µg/ml) in each sample. After 24 hrs cells were collected and rinsed twice with PBS (pH 7.4) and observed by fluorescence microscopy.

3. RESULTS AND DISCUSSION

3.1 Preparation of Solid lipid nanoparticles

The Telbivudine loaded solid lipid nanoparticles were prepared by emulsification- Sonification method with slight modification. Different types of lipids, type of surfactant and concentration of surfactant were selected as variables to optimize the formulation.

3.2 Optimization of Solid Lipid Nanoparticles

Solid Lipid nanoparticles were prepared by emulsification method and hence size, size distribution and entrapment efficiency of drug plays a important role in optimizing the formulation. The particle size of prepared SLN should be in nano range, PDI is important aspect as SLN may show aggregation so it may affect the homogeneity of formulation and entrapment efficiency is important for uniformity of dose. So, optimization was done on basis of Particles Size, PDI and entrapment efficiency.

Table 3: Entrapment Efficiency and Particle Size of prepared Batches

Formulation Code	% Entrapment Efficiency	Particle Size (nm)	PDI
SLN-1	75.89	1116	0.83
SLN-2	77.23	930.3	0.496
SLN-3	78.45	588.8	0.633
SLN-4	72.56	322.6	0.409
SLN-5	74.76	786.1	0.823
SLN-6	77.23	430.7	0.444
SLN-7	77.97	208.8	0.366
SLN-8	82.72	443	0.328
SLN-9	84.22	488.2	0.425
SLN-10	Separation of Phases	Separation of Phases	Separation of Phases
SLN-11	Separation of Phases	Separation of Phases	Separation of Phases
SLN- 12	Separation of Phases	Separation of Phases	Separation of Phases
SLN- 13	75.24	435.6	0.678
SLN- 14	77.59	458.3	0.754
SLN- 15	79.23	496.8	0.486
SLN- 16	78.72	623.5	0.548
SLN- 17	81.22	689.2	0.825
SLN- 18	83.25	724.6	0.854
SLN-19	77.45	1189	0.878
SLN-20	78.34	967.3	0.596
SLN-21	79.23	623.34	0.636
SLN-22	75.56	374.45	0.523

SLN-23	76.78	823.34	0.867
SLN-24	78.34	530.7	0.487
SLN-25	78.56	328.8	0.523
SLN-26	84.34	489	0.567
SLN-27	86.34	568.2	0.498
SLN-28	Separation of Phases	Separation of Phases	Separation of Phases
SLN-29	Separation of Phases	Separation of Phases	Separation of Phases
SLN- 30	Separation of Phases	Separation of Phases	Separation of Phases
SLN- 31	77.34	498.2	0.678
SLN- 32	78.23	567.2	0.798
SLN- 33	80.12	589.2	0.534
SLN- 34	79.34	629.8	0.598
SLN- 35	82.45	734.2	0.86
SLN- 36	84.56	786.4	0.898

3.3 Characterization of optimized formulations

On basis of particle size and entrapment efficiency formulations were optimized. The results showed that all the three variables had significant influence on size and entrapment efficiency.

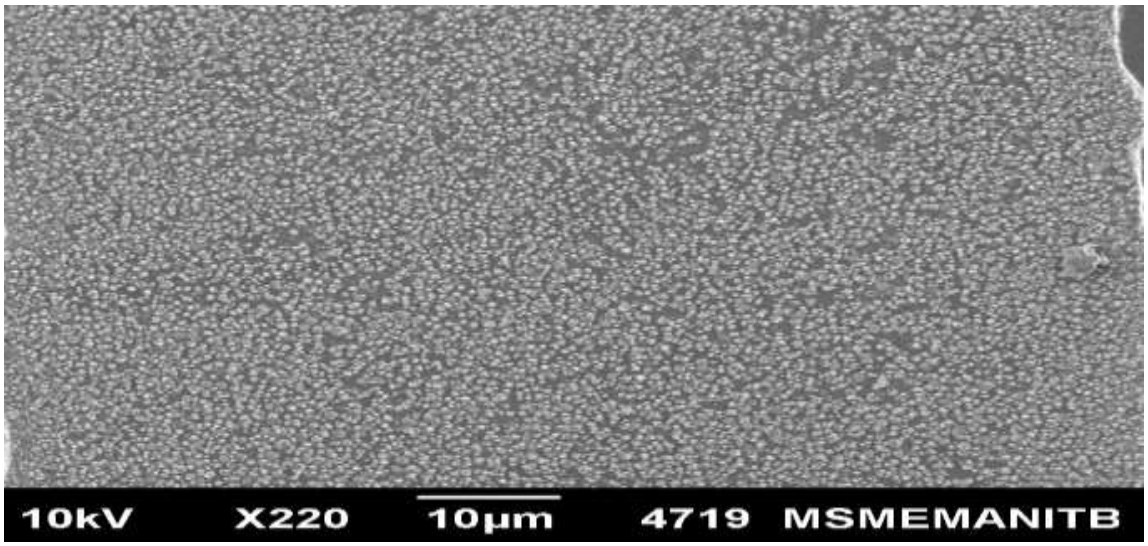
3.3.1 Zeta Potential

It is one of the most important parameters to check stability of SLN as zeta potential is measure

3.3.2. Particle Morphology

Morphology of the solid lipid nanoparticles was examined by SEM and is shown in Figure 4. Telmivudine loaded solid lipid nanoparticles have an almost spherical shape and smooth surface because these characteristics depend on the lipid.

Fig. 4: Scanning Electron Micrograph of Solid Lipid Nanoparticles of Telmivudine.



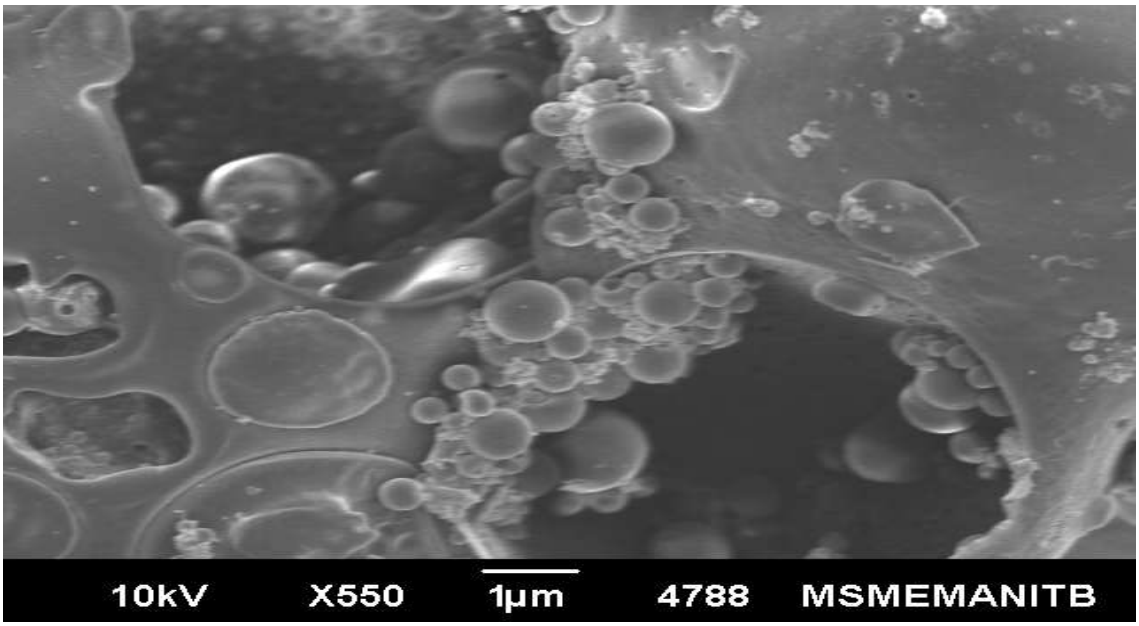


Fig. 5: Scanning Electron Micrograph of Galactose modified Solid Lipid Nanoparticles of Telmivudine.

3.4 Preparation of Galactose modified SLN (GmSLN)

The Coupling of Galactose (GA) was done by method using Xia et al 2019 with slight modifications. The Prepared SLN Suspension (2 ml) was taken in Beaker and Then 1 ml (2 mg/ml) galactose+ 1 ml of Epichlorohydrin as linker was dropped to SLN suspension mixed using magnetic stirrer for 4 h to fabricate GA-modified SLN (GmSLN). The FTIR Studies revels that modification of Galactose on the SLN.

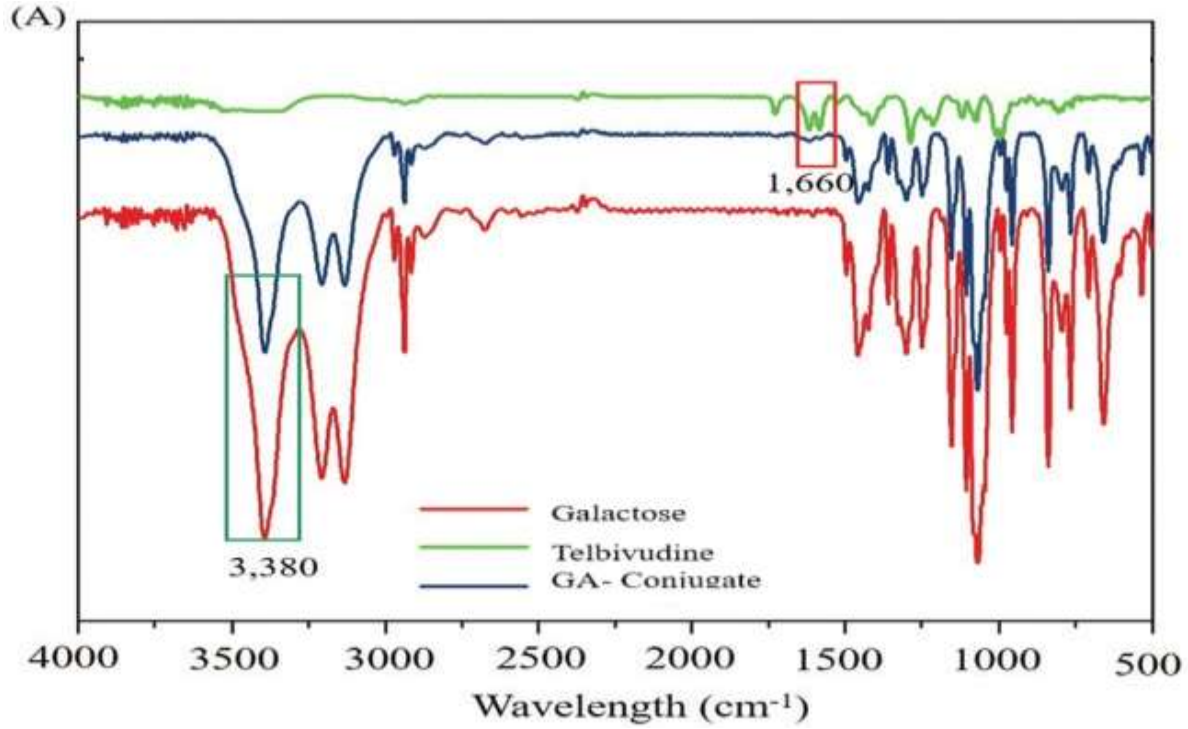


Figure 6: Overlay FT-IR Spectrum of Drug (Telmivudine), Galactose (GA) and Conjugate

3.5 *In-vitro* Drug Release

Figure 7 shows the *in-vitro* release profile of Telmivudine from nanoparticulate dispersion. In the initial 2 hours, the drug release was less than 8%, probably because of the slow diffusion of drug from the lipid but it shows controlled release until 24 hours. The prolonged drug release could be attributed to embedment of drug in the solid lipid matrix.

Comparing the drug release from uncoated nanoparticulate dispersion and Galactose Coated Nanoparticles, the release of Telmivudine was slower from the coated formulation, 51.22% as compared with uncoated nanoparticulate dispersion, 55.48% at the end of 24 hours. This result was probably due to the release-retarding effect of the coating by Galactose.

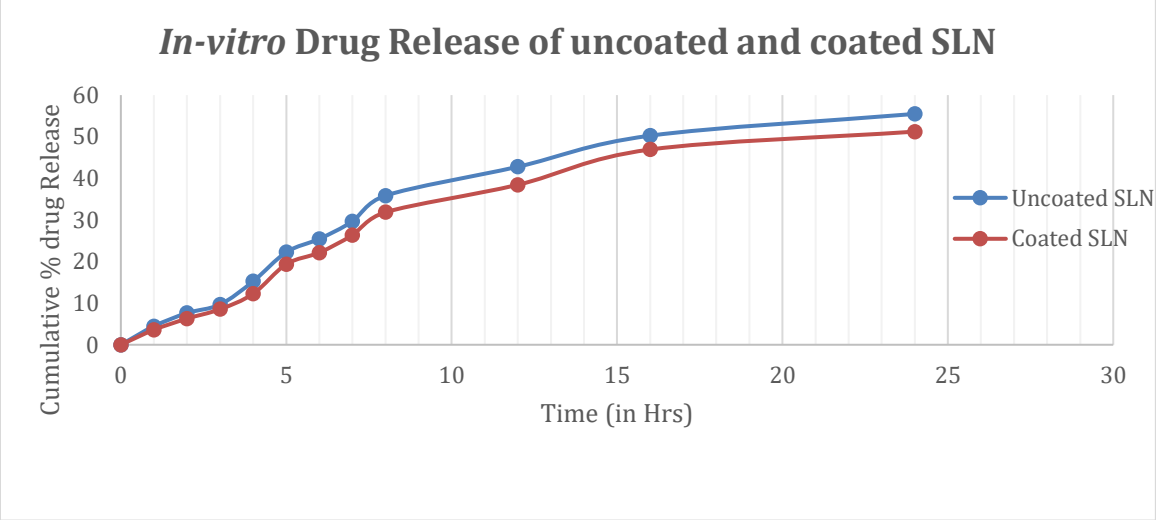


Fig.7: *In-Vitro* Release of Uncoated SLN and Galactose Coated Nanoparticulate Dispersion.

3.6 Hepatoprotective activity against DCFH-hepatotoxicity

HepG2 cells were seeded in a 96-well flatbottom plate and grown overnight. The concentration of DCFH that caused 50% inhibition of cell proliferation (IC50: 100 µg/ml), was used as a cytotoxic dose (Al-Yahya et al., 2013; Arbab et al., 2015), prepared in DMSO. The culture monolayer was replenished with RPMI-1640 containing 100 µg/ml DCFH plus a dose of plant fraction (25, 50, 100 and 200 µg/ml), including untreated as well as DCFH only-treated controls. All samples were in triplicate. The treated cells were incubated for 48 h at 370 C followed by MTT assay. The optical density (OD) was recorded at 570 nm in a microplate reader (BioTek, ELx800) and the data analyzed.

In vitro hepatoprotective effect of SLN and GA modified SLN against DCFH-induced hepatotoxicity was investigated on cultured cells using MTT assay. DCFH-toxicated cells were recovered to about 67% and 86% upon treatment with 200 µg/ml of SLN and GA modified SLN, respectively.

3.7 Cellular Uptake Studies

The Hepa-1c1c7 cells were seeded onto a glass-bottomed culture dish (MatTek Corp., Ashland, MA) and incubated with fresh a-MEM supplemented with 5% feral bovine serum (biowest, Nuaille´, France) for 24 h prior to treatment. The cells were treated with different drug loaded SLN in the medium. After the incubation, the cells were fixed with 4% paraformaldehyde and 0.2% picric acid in 100 mM sodium phosphate buffer (pH 7.2) overnight. All the test samples were prepared by adding FITC dye (100µg/ml) in each sample. After 24 hrs cells were collected and rinsed twice with PBS (pH 7.4) and observed by fluorescence microscopy.

The cellular uptake of SLN and Modified SLN was detected by fluorescence in Hepa-1c1c7 cells under the confocal scanning microscope. Green fluorescence was detected at over 50 µM of drug (Fig. 7). The time-dependent uptake of drug at 50 µM was also monitored, the intensity of fluorescence increased time-dependently (Fig. 8). After 15 min, apparent fluorescence was observed to the cells. Taken together, the fluorescence microscope is able to monitor the uptake of drug in Hepa-1c1c7 cells.

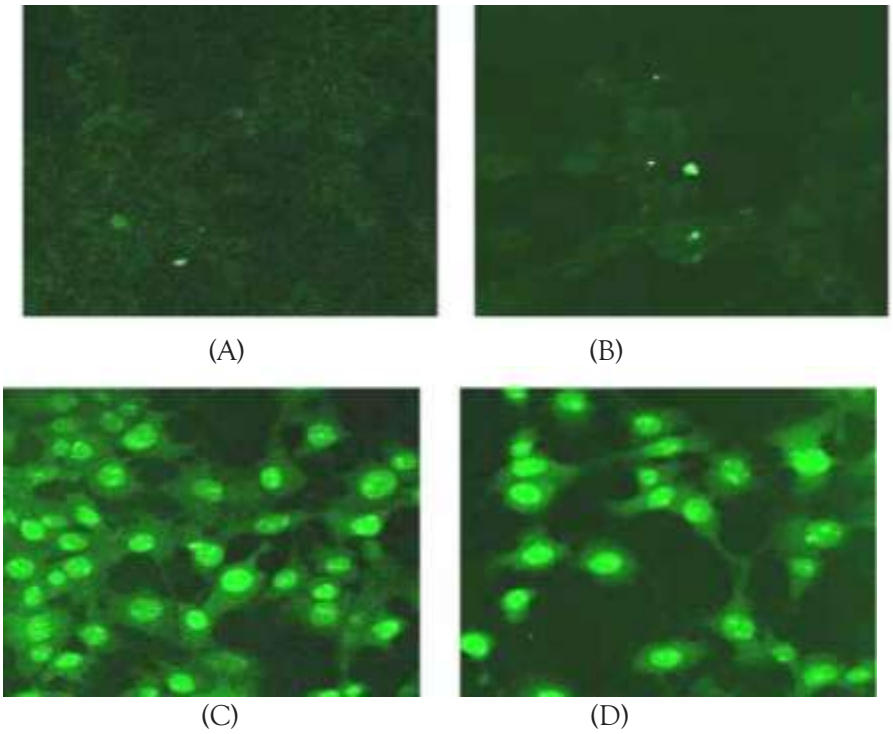


Figure 7: Cellular uptake of drug visualized by a fluorescence microscope in Hepa-1c1c7 cells. (A) 10 μM, (2) 25 μM, (3) 50 μM and (4) 100 μM for 70 min.

The time dependent cellular uptake is also studied which reveals that GA- modified system shows better uptake in less time as compared to Plain SLN which proves that modification increases uptake in hepatocyte cells after modification with galactose.

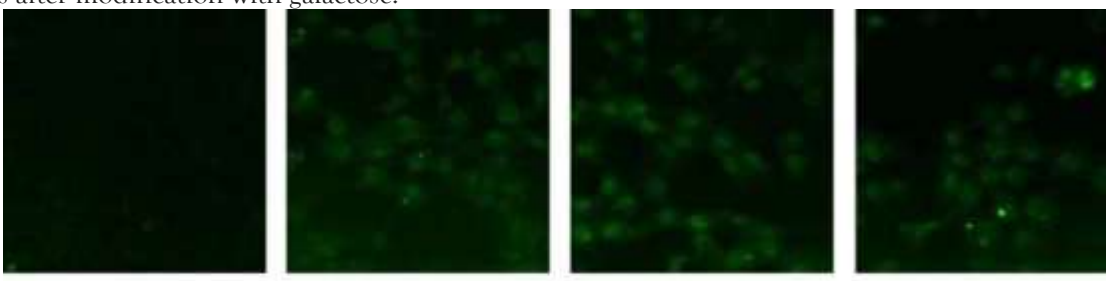


Figure: 8: Time-dependent cellular uptake of SLN in Hepa-1c1c7 cells.
(A) 4 hr (2) 8 hr, (3) 12 hr and (4) 24 hr

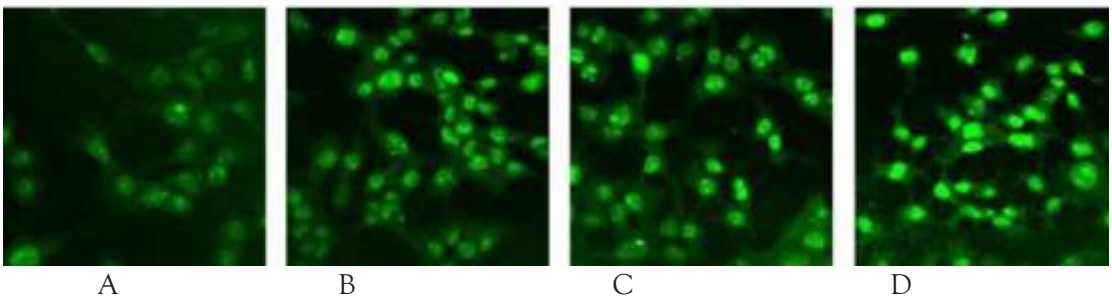


Figure: 9: Time-dependent cellular uptake of GA modified SLN in Hepa-1c1c7 cells.

(A) 4 hr (2) 8 hr, (3) 12 hr and (4) 24 hr

4. CONCLUSION:

The aim of this research work to target hepatocytes cells which are affected in hepatitis b infection. Telbivudine is antiviral drug which was used effectively against hepatitis b infection. In Present research work , Galactose coated Solid lipid nanoparticles were prepared Containing antiviral drug to treat hepatitis b infection.

In Present work, the galactose coated SLN Shows better uptake than normal SLN which proves that liver cells are better targeted than Plain SLN. The drug is only available in oral dosage form and hence optimized formulation can be alternative to present dosage form with reduced dose and dosing frequency.

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