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Abstract

Now a day, millions of people suffer from chronic Hepatitis B Virus globally. Presently, no well-established treatment is available for the chronic Hepatitis B virus infection; the available treatment is based on host mediated immunological control and reduction of HBV-DNA levels in blood serum. There is a constant demand for new and improved vaccines, scientist have continually develop a new vaccine technology. In this research article given a different approach for the preparation of Hepatitis B vaccine, utilizing a novel drug delivery system. In this regard developed a Hepatitis B antigen loaded polymeric and lipid particles. Prepared particles were further characterized for there particles size, entrapment efficiency, morphology and in vitro release. Further, compare the formulation on the basis of characterization. Results indicated that, polymeric particles showed high entrapment efficiency, and better release. This formulation is suitable for further clinical study.

Keywords: Nanoparticles, vaccine delivery, polymer, lipid.

INTRODUCTION

The worldwide problem of Hepatitis B is the common serious liver necroinflammatory infection, and it is referred as silent killer initiated by Hepatitis B Virus (HBV) which slowly damages the liver. According to the World Health Organization (WHO), Hepatitis B is a potentially life-threatening liver infection caused by Hepatitis B virus. Viral infections causes acute and chronic inflammatory diseases of liver and, in some cases, develops into serious complication such as hepatocellular carcinoma or liver cirrhosis or chronic liver disorders. Now a days, millions of people suffer from chronic HBV globally [1, 2]. Hepatitis B generally affects the healthy workers, so that, it requires a nontoxic and effective vaccine.

Those people infected by birth or in early childhood, are having higher chances of chronic Hepatitis B virus because the immune system is not fully developed [3, 4]. Chronic Hepatitis B virus infection is directly proportional to the age and time of first exposure. When cellular immunity is pharmacologically restrained, Hepatitis B virus grows rapidly and replicates continuously at high levels, resulting in chronic cytological abnormalities or inflammation in liver tissues [5, 6]. Lack of T-cell adaptive immune response and specific humoral immune response is another reason of chronic HBV infection (WHO 2014). As per literature, for the generation and production of protective immune system, both the adaptive as well as innate immune systems are required [7].

The most recent long term potential approach to eliminate the virus is immunotherapy. It works to stimulate an adaptive immune response through immunization or vaccination or inoculation. Vaccine is made by modified or killed microorganisms like bacteria or viruses, which develops resistance for specific disease. It does not promote the cause of disease but builds a protective immune system, which continuously protect against the disease [8, 9]. The immunization or vaccination is not the treatment and cure for the chronic infection and not even the treatment of infection.

The more appropriate approaches of curing Hepatitis B virus infection is manipulation of host immune system and clearance of virus but manipulation of host immune system is very difficult. Some time it creates major problems such as cirrhosis, liver cancer and finally death [10].

To overcome these problems and limitations of currently available therapies, a new nanovaccine or nanoparticulate system against the Hepatitis B Virus infection was developed.

In the past decades, the application of nanotechnology in virology has been increased tremendously. Nanoparticles having different compositions, various sizes, and surface morphology properties were developed [11]. Nanoparticles based approach is revolutionizing the diagnosis of disease as well as the delivery of active substance for the treatment and prevention of disease [12].

MATERIALS AND METHODS

Poly(d,l-lactide-co-glycolide, PLGA; L/G = 50/50, MW 38,000–54,000), provide as a gift sample by EVONIK industry, Polyvinyl alcohol (PVA) MW:72,000, were purchased from Sigma-Aldrich (St,Louis MO, USA). Hepatitis B Antigen was provided as gift sample from Serum Institute Pune, Dichloromethane was procured from Thermo Fisher Scientific-Gibco (Waltham, USA.) India Pvt. Ltd. Methanol (HPLC grade), Tristearin, span 80 and Tween-80 were purchased from Himedia Pvt. Ltd., Mumbai, India. Chloroform, and ethanol was supplied by SD Fine-Chem Ltd. (Mumbai, India), Distilled or purified deionised water was used throughout experiment and obtained from Millipore Water Purifier System (Milli-Q water gradient, resistivity ≥ 18 Ω cm). All buffer salts and other chemicals used in analytical grade.

HPLC analytical method

A reverse-phase high-performance liquid chromatography (HPLC) system with photo diode detector was used for the in vitro analysis of HBsAg. The following HPLC condition was preferred for the analysis [13].

Column: C18 reverse phase, 250mm X 4.5mm,

Mobile phase: methanol- phosphate buffer (pH 7.4) (50:50 % V/V)

Flow rate: 1.0 mL/min

Column temp: 25±2 °C

Detector: PDA (306 nm)

λ max: 280 nm

Fabrication of Antigen (HBsAg) loaded PLGA nanoparticles

Antigen-loaded NPs were prepared by double emulsion solvent evaporation method. Saline (100 µL) containing Antigen was emulsified with 2.5 mL of dichloromethane (DCM, organic phase), containing PLGA (35 mg), and sonicate for 2 min, using span 80 as emulsifier, to obtained primary W/O emulsion. The primary emulsion was again emulsified in 1%, W/V PVA and homogenized for 20 min, to find W1/O/W2 emulsion. Then evaporate the organic solvent at room temperature. NPs were washed twice with Milli-Q water and lyophilized [14].

Fabrication of Antigen (HBsAg) loaded Solid Lipid Nanoparticles (SLN)

SLN were prepared by solvent injection method. Briefly, at room temperature tristearin (50 mg) was dissolved in 3.0 mL of acetone. Separately, 2.5% w/v lactose monohydrate was added to 4 mL of saline solution of antigen and then this solution was added to aqueous phase containing Tween-80 (0.5% v/v). After that organic phase was injected rapidly through an injection needle into the continuously stirred aqueous phase. Subsequent dispersion was then filtered and collect [15, 16].

Fabrication of Antigen (HBsAg) loaded liposomes

Elastic liposomes were prepared by ethanolic injection method. Ethanolic solution of soya phosphatidyl choline was mixed with Span 80 (86:14% (w/w)) in phosphate buffer saline containing HBsAg solution and the suspension was pushed through a series of 0.45, 0.22m filters to obtained a liposome [17].

Particle size and entrapment efficiency

The average size (Z-average) of the nanoparticles was determined by Beckman Coulter particle size analyzer. A concentrated sample was prepared in milli-Q water sonicated on an ice bath. All measurements were performed in triplicate.

The entrapment efficiency estimated by using an in-direct procedure. In this method, prepared sample was centrifuged at 18,000 rpm for 15 min. (eppendorf tube) An aliquot of the supernatant was collected, and proceed for the for HPLC analysis [18]. The following equation was used:

$$\text{Encapsulation Efficiency} = \frac{\text{Weight of drug in nanoparticles}}{\text{Initial amount of drug}} \times 100$$

Morphology study

Transmission Electron Microscopy (SEM): The sample was negative stained with phosphotungstic acid (2% w/v) for TEM analysis. The internal morphology of nanoparticles was studied by depositing 10 µL of each sample on copper grids using transmission electron microscopy (Tenai G^2 20 Twin, FEI Company, Netherland).

In vitro release of HBsAg- loaded Nanoparticles

The in vitro release of HBsAg from nano shell was performed in phosphate buffer saline at pH 7.4 using Dialysis bag Method (DM). 2 mL of sample were introduced into a dialysis bag then sealed properly and placed in a larger container containing phosphate buffer saline, at 37 ^{0}C, under gentle agitation [19]. The sample were withdrawn at predetermine intervals and equal volume of fresh buffer was replaced and further processed for HPLC analysis.

RESULTS

The calibration curve of HBsAg was linear over the conc. range of 1-25 µg/mL at retention time of 4.8±0.1 min. The correlation coefficient (R^2) was found to be 0.9998. The selected method was precise and accurate with linear response (Figure 1).

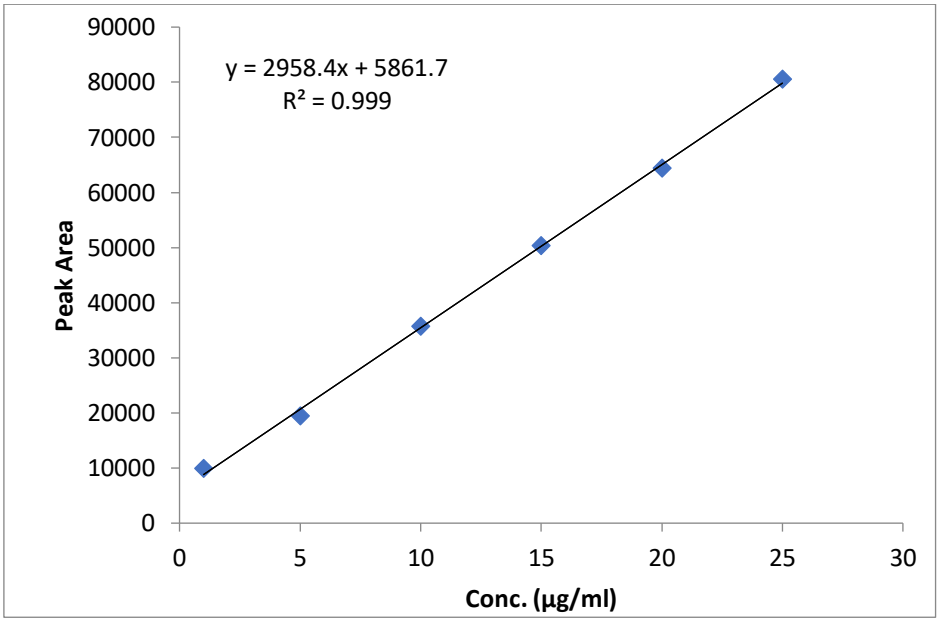

Figure 1: Standard Calibration Curve of HBsAg

All these three formulations like nanoparticles, solid lipid nanoparticles and liposomes were successfully prepared. Prepared formulations were proceeded for the characterization. Table 1

represent the physical characterization of HBsAg. Morphology of different formulation is presented in figure 2, which showed the spherical shape all formulation batches.

Table 1: characterization of different formulation of HBsAg

Parameters	Formulation		
	Nanoparticles	**Solid lipid nanoparticles**	**Liposomes**
Particles/ vesicular size	224 nm	187 nm	149 nm
% entrapment efficiency	86 ± 23 %	63±62 %	61±56 %
Shape of particle/vesiculas	Spherical	Spherical and lamellar	Spherical and lamellar
% cumulative release in 24 hr	29 ± 83 %	41 ± 53 %	62 ± 33 %

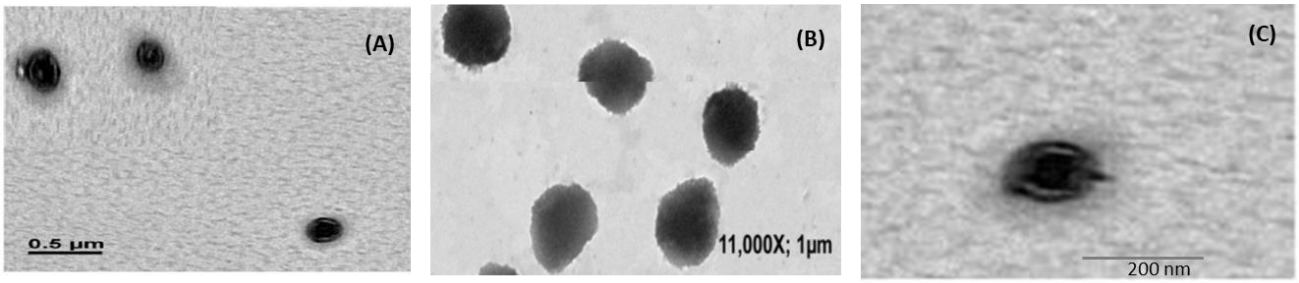

Figure 2: TEM image of formulation (A) HBsAg loaded nanoparticles (B) HBsAg loaded solid lipid nanoparticles (C) HBsAg loaded liposomes.

DISCUSSION

The effective delivery of the therapeutic molecules requires easy crossing across the barrier, reaching the target site and fighting against the disease-causing agents. Polymeric nanoparticle system is more beneficial and effective in comparison to traditional system. Nano size of particles could improve degradation as well as diffusion and shows time dependent controlled release of drug by the diffusion of encapsulated materials or degradation of outer shell. Nanometric sizes of particles influence the rate of absorption, distribution, metabolism, and excretion. Polymeric nanoparticles may allow binding drug at target receptor site and influences the receptor activity [20, 21].

The nanoformulations have greater efficiency and safety in comparison to conventional products. Nano formulation is easy to formulate and capable to treat various diseases or

infections. Nanoparticles work as smart delivery system *i.e*. it is having the capability for to programmed detection, self-regulation and delivery of therapeutic molecules at the target site. It also monitors the effects of delivered molecules, pharmaceutical drugs, food supplements, chemicals, nutraceuticals and vaccines [22]. Super paramagnetic nanoparticles, nanoshells and quantum dots have also been used to identify, trace and destroy infectious organisms or diseased cells.

The key feature of this nanoparticles formulation in comparison to solid lipid nanoparticles and liposomal. The biodegradable nanoparticles have been used as effective carriers of HBAg delivery. The PLGA nanoparticles are successfully developed to improve the systemic action. The nanoparticles prepared by using double emulsion solvent evaporation technique. It has nanometre size, high entrapment efficiency and good release profile. The formulation development achieves a better superiority and high shearing strength. Some factors such as amount of polymer, concentration of stabilizer aqueous organic phase ratio and speed of homogenizer significantly influence both the particle size and entrapment efficiency of formulation.

In case of SLN utilized a solvent injection technique for formulation which found to depend on rapid diffusion of solvent across the solvent lipid interface with the aqueous phase. The use of emulsifier reduces the surface tension between aqueous and organic phase and leads to the formation of smaller solvent droplets, thus, smaller size of particles was obtained. Moreover, with increasing lipid concentration, viscosity of organic phase increased, which causes slower diffusion of the organic solvent in the outer phase. Thus, chances of larger particle size and lower entrapment [23]. Increase in surfactant concentration led to decrease in particle size, means at a lower Tween-80 concentration, higher particle size obtained.

Further, in case of liposome, ethanol present in formulation might be intercalated in lipid bilayers and hence did not exist in free form to degrade the entrapped antigen. Ethanol interacts with lipid molecules in the polar head group region. The intercalation of ethanol into the polar head group environment can result in chances of enhancement in the membrane permeability. In conclusion, in the present study HBsAg-loaded nanoparticles showed greater entrapment, nano sized and good release pattern. Therefore, prepared formulation aspect to produce cellular and humoral immune response in clinically.

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Conflict of interest

The authors have no conflict of interest.

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