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FORMULATION OPTIMIZATION AND *IN VITRO* RELEASE STUDY OF DIDANOSINE LOADED BOVINE SERUM ALBUMIN NANOPARTICLES

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ABSTRACT

Albumin is an attractive macromolecular carrier and widely used to prepare nanospheres and nanocapsules, due to its availability in pure form and its biodegradability, nontoxicity and nonimmmunogenicity.In the present work Didanosine loaded bovine serum albumin nanoparticles were prepared by desolvation method. Optimization of the formulations was done by selecting four process variables: stirring speed, pH of solution, polymer concentration and amount of cross linking agent (glutaraldehyde). The particle sizes of nanoparticles were found in the range of 50.5-109.1 nm, zeta potential -23.5, drug loading was 36.63 µg per mg of nanoparticles, entrapment efficiency was 80.87%, and % cumulative release was 82.13% after 48 hrs.

KEYWORDS : *Didanosine, Nanoparticles, Bovine Serum Albumin, Glutaraldehyde*

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INTRODUCTION

Colloidal drug carriers include micelles, emulsions, liposomes and nanoparticles (nanospheres and nanocapsules). The aim in using colloidal carriers is generally to increase the specificity towards cells or tissues, to improve the bioavailability of drugs by increasing their diffusion through biological membranes and/or to protect them against enzyme inactivation. Moreover, the colloidal systems allow access across the BBB of non-transportable drugs by masking their physicochemical characteristics through their encapsulation in these systems [1].

The term "nanoparticle" may be defined as a submicron drug carrier system, generally (but not necessarily) of polymeric nature (the polymer used may be or not biodegradable). Thus, this term is somewhat general since it does not take into account the morphological and structural organization of the polymer. In this respect, "nanosphere" is used to identify a nanoparticle system with a matrix character and constituted by a solid core with a dense polymeric

network. In contrast, "nanocapsules" are formed by a thin polymeric envelope surrounding an oil-filled cavity. Nanocapsules may thus be considered as a "reservoir" system. Practically, the nanoparticles have a size around 200 nm and the drugs or other molecules may be dissolved into the nanoparticles, entrapped, encapsulated and/or adsorbed or attached. These systems are attractive because the methods of preparation are generally simple and easy to scale-up. Nanoparticles can be made from a broad number of materials such as poly (alkylcyanoacrylates) (PACAs), polyacetates, polysaccharides and copolymers [2,3].

The advantages of using nanoparticles as a drug delivery system include the following [4]

1. Particle size and surface characteristics of nanoparticles can be easily manipulated to achieve both passive and active drug targeting after parenteral administration.
2. They control and sustain release of the drug during the transportation and at the site of localization, altering organ distribution of the drug and subsequent clearance of the drug so as to achieve increase in drug therapeutic efficacy and reduction in side effects.
3. Controlled release and particle degradation characteristics can be readily modulated by the choice of matrix constituents.
4. Site-specific targeting can be achieved by attaching targeting ligands to surface of particles or use of magnetic guidance.
5. The system can be used for various routes of administration including oral, nasal, parenteral, intra-ocular etc.

Methods of preparation of nanoparticles

(A)Emulsification Solvent Evaporation The solvent evaporation method was described by Niwa *et al* and has since been widely used to prepare particles from a range of polymeric materials, particularly PLA and PLGA [5].

(B) Interfacial Polymer Deposition (IPD) Method The IDP method was first described by Fessi *et al* [6] the technique involves addition of polymer, dissolved in a water miscible solvent (usually acetone) into an aqueous non solvent under stirring.

(C)Spray Drying In the classical spray drying technique the polymer and drug are dissolved in an organic solvent and sprayed through a fine nozzle. Solid, spherical particles form on the immediate evaporation of the solvent.

(D)Salting Out The technique involves the preparation of particles by an emulsification technique but avoids the use of chlorinated solvents.

(E)Supercritical fluid expansion method Recently the field of supercritical fluids has been investigated as an approach to the preparation of sub micron sized particles [7]. The rapid expansion of supercritical solutions consists in saturating a supercritical fluid with the substrate(s), then depressurizing this solution through a heated nozzle into a low pressure chamber in order to cause an extremely rapid nucleation of the substrate in form of very small particles or fibres, or films when the jet is directed against a Surface that are collected from the gaseous stream[8].

(F)Complex Coacervation Complex coacervation is a phase separation process that spontaneously occurs when two oppositely charged polyelectrolytes are mixed in an aqueous solution[9-11].

Albumin is an attractive macromolecular carrier and widely used to prepare nanospheres and nanocapsules, due to its availability in pure form and its biodegradability, nontoxicity and nonimmmunogenicity. On the other hand, albumin nanoparticles are biodegradable, easy to prepare in defined sizes, and carry reactive groups (thiol, amino, and carboxylic groups) on their surfaces that can be used for ligand binding and/or other surface modifications and also albumin nanoparticles offer the advantage that ligands can easily be attached by covalent linkage[12].

The objective of present investigation was to prepare controlled release nanoparticles by desolvation and subsequent cross-linkage of didanosine using bovine serum albumin as a polymer. A systematic investigation concerning the influence of stirring speed on particle size and

amount of albumin, pH, amount of cross-linking agent on drug loading efficiency, release properties *in vitro* was carried out.

MATERIALS AND METHODS

Didanosine was a gift sample from Cipla Ltd, Bovine Serum Albumin and Glutaraldehyde were purchased from Sigma-aldrich, All other chemicals were of analytical grade.

Preparation of didanosine nanoparticles

Nanoparticles of didanosine were prepared by desolvation method by optimizing formulation variables such as speed, polymer concentration, pH, amount of glutaraldehyde.For the preparation of nanoparticles, didanosine (10 mg) was dissolved in 5 ml of distilled water, in this drug aqueous solution bovine serum albumin was added and titrated to adjust pH. This solution was incubated for 2 hours. Afterward 7.5 ml of ethanol was added (0.5 ml/min) in the aqueous albumin solution under magnetic stirring. After the desolvation process, 8% glutaraldehyde was added to crosslink the desolvated bovine serum albumin nanoparticle and the cross linking process was performed under stirring of colloidal suspension over a time period of 24 hours.

Separation of Nanoparticles

Finally the resulting nanoparticles were purified and obtained by centrifugation. Microparticles were precipitated by centrifugation (15,000 rpm, 2 min) and then discarded. Nanoparticles were separated by three cycles of centrifugation (17000 rpm, 20 min) and redispersion of the pellet to the original volume in distilled water containing 2% mannitol, then freezed and finally freeze dried for 24 hours.

CHARACTERIZATION OF NANOPARTICLES

Surface Morphology

Scanning electron microscopy was conducted (JEOL JSM – 6380LA) to characterize the surface morphology of nanoparticles. Powders were deposited on carbon conductive double sided tape and dusted to remove the excess and sputtered with platinum for 1 min.

Size and Size Distribution

The average diameter and size distribution study of BSA nanoparticles were done by dispersion of each lyophilized formulation (5 mg) in distilled water (10 ml) with manual shaking for 10 minutes. The size distribution profile of BSA nanoparticles was determined by photon correlation spectroscopy (Zetasizer nanoseries Nano-ZS-90, Malvern instruments, UK)

Yield of Nanoparticles

The yield of different nanoparticles formulation was calculated as the percentage of weight of lyophilized nanoparticles relating to the initial of raw material (BSA, Drug).

$$\%\ \text{YIELD} = \frac{\text{AMOUNT OF DRIED NANOPARTICLES}}{\text{AMOUNT OF POLYMER + DRUG}} \times 100$$

Drug-Polymer Interaction (FT-IR STUDIES)

Transmission infrared spectra of polymer (bovine serum albumin) and BSA nanoparticles loaded with didanosine were measured by using Fourier-transform infrared spectrometer (JASCO-470 plus) in wavelength region of 4000-400 cm^{-1}. The sample was mixed with KBr and placed in to sample holder. The sample was placed in the light path and spectrum was recorded**.**

Drug Loading and Entrapment Efficiency

Lyophilized nanoparticles (10 mg) were dispersed in 10 ml of phosphate buffer saline pH-7.4. Afterward for complete liberation of entrapped drug 0.1 ml of trypsin (1mg/ml) was added and the resulted solution was incubated at 37^0C for 24 hours. Later total drug concentration was determined spectrophotometrically at 249 nm.

$$\text{DRUG LOADING} = \frac{\text{AMOUNT OF DRUG IN NANOPARTICLES (µg)}}{\text{AMOUNT OF NANOPARTICLES (mg)}}$$

$$\%\ \text{ENTRAPMENT EFFICIENCY} = \frac{\text{AMOUNT OF DRUG IN NANOPARTICLES}}{\text{AMOUNT OF INITIAL DRUG ADDED}} \times 100$$

Zeta Potential

Zeta potential is an abbreviation for electrokinetic potential in colloidal systems. The significance of Zeta potential is that its value can be related to the stability of colloidal dispersions.

In vitro Release

Drug release from didanosine nanoparticles were determined as follows. The mass of nanoparticles equivalent to 2 mg of drug were re-dispersed in mammalian ringer solution and placed in a dialysis membrane bag with a cut-off of 25 kDA. Then the DID-BSA-NP contained bag was tied and put in to 40 ml of mammalian ringer's

solution. The entire system was incubated at 37^0C under stirring at 100 rpm. At designated time intervals, 3ml of the release medium was removed and replaced with the same volume of fresh MRS solution to maintain the sink condition. The amount of didanosine in the release medium was determined spectrophotometrically at 249 nm.

OPTIMIZATION OF PROCESS VARIABLES

There are number of variables observed in the preparation of nanoparticles. All these have their individual effect on the properties of nanoparticle like particle size, zeta potential, Polydispersity index, loading efficiency and release profile.

1) Effect of Stirring Speed
2) Effect of pH
3) Polymer Concentration
4) Amount of Cross linking agent (8% Glutaraldehyde)

RESULTS AND DISCUSSION

Scanning electron microscopy was performed to characterize the morphology of nanoparticles. The particle with magnification of 10000 and 30000 respectively. The shape of the nanoparticles demonstrated in SEM (Figure 1) is spherical. The sizes of fabricated nanoparticles were absolutely less than 500 nm.Smaller particles have higher surface area/volume ratio, which makes it easier for encapsulated drug to be released from the nanoparticles via surface erosion

The stirring speed was varied from 100 to 800 rpm while keeping other processing parameters. On increasing speed decrease in mean diameter was observed. These trends occur because at high speed distribution of desolvating agent (ethanol) occurs uniformly which inhibit aggregate formation.

DRUG-POLYMER INTERACTION (FT-IR STUDIES)- Infrared spectra of proteins exhibit a number of amide bands which represent different vibrations of the peptide moiety. As shown in Figure-2,3the peak position of amide I band and amide II band moved from 1685 cm^{-1} to 1655 cm^{-1} and 1560 cm^{-1} to 1539 cm^{-1} after formation of didanosine loaded nanoparticles. The changes in these peak position indicate that drug (Didanosine) may interacted with BSA and causing change in secondary structure of BSA. The peak position of amino group in BSA moved from 3459 cm^{-1} to 3277 cm^{-1} after formation of nanoparticles indicate cross linking of amino groups by gluteraldehyde.

DRUG LOADING AND % ENTRAPMENT EFFICIENCY

Effect of pH- The pH of solution was varied from 5.5 – 8.5 while keeping other processing parameters constant. At pH-5.5 precipitate was formed. At high pH value BSA was charged (zwitter ion form $–COO^-$ and $–NH_3^+$) therefore it could ionically interact with didanosine. So due to high interaction between drug and polymer at high pH drug loading and entrapment efficiency was increased.

Effect of polymer concentration -Polymer concentration was varied from 20 mg/ml to 100 mg/ml while keeping other processing parameters constant (Table-3). It was observed that by increasing polymer concentration drug loading and entrapment efficiency was increased up to 60 mg/ml, This may be due to adsorption or interaction with polymer, so increase in polymer enhances it's interaction with drug. Afterward increase in polymer concentration decreases drug loading due to increase in amount of nanoparticles.

Effect of amount of cross linking agent (8% glutaraldehyde)- Amount of glutaraldehyde (8%) was varied from 0.6 µl/mg of BSA to 1.4 µl/mg of BSA while keeping other processing parameters constant (Table-4). It was observed that increase in amount of glutaraldehyde decreases drug loading and entrapment efficiencybecause the hydrophobic tendency of protein particles induced by glutaraldehyde might also hinder the constantly encapsulation of the hydrophilic water soluble drug.

The **Zeta potential** indicates the degree of repulsion between adjacent, similarly charged particles in dispersion. A value of 25 mV (positive or negative) can be taken as the arbitrary value that separates low-charged surfaces from highly-charged surfaces. The Zeta potential of nanoparticles was found -23.5 mV.

***IN VITRO* RELEASE STUDIES**

Effect of amount of glutaraldehyde (8%)-Amount of gluteraldehyde (8%) was varied from 0.6 µl/mg of BSA to 1.4 µl/mg of BSA while keeping other processing parameters constant (Table-4). As shown in Figure-4, in 24 hours, 49% drug released from nanoparticles cross linked with 0.6 µl per mg of BSA. Then the drug released even more slowly with increased amount of gluteraldehyde used 1.0 µl per mg of BSA that is 17% in 24 hours. The sustained release effect could not be enhanced significantly even in cross linked 1.4 µl 8% gluteraldehyde per mg of BSA. This might be explained by the limited surface free amino group on BSA nanoparticles. The further augmented cross-linkage of albumin matrix seems not significantly affect the release of didanosine from BSA nanoparticle cross linked with 1 µl per mg of BSA.

Finally the NP-17 was considered as optimized formulation which was prepared at stirring speed 700 rpm, pH-8.5, polymer concentration 60 mg/ml and amount of glutaraldehyde 0.6 µl per mg of BSA. The particle size nanoparticles were found between 50.5-109 nm, zeta potential -23.5, drug loading was 36.63 µg per mg of nanoparticles, entrapment efficiency was 80.87%, and % cumulative release was 82.13% after 48 hrs.

Table-1 Optimization of Stirring Speed

BATCHCODE	STIRRING SPEED (rpm)	SIZE (nm)	PDI	% YIELD	DRUG LOADING (µg/mg)
NP-1	100	200.7	0.307	69.20	12.99
NP-2	200	107.5	0.374	69.80	12.84
NP-3	300	115.5	0.462	69.21	12.85
NP-4	400	105.4	0.431	69.91	12.76
NP-5	500	83.98	0.378	70.20	12.48
NP-6	600	82.97	0.297	71.21	12.39
NP-7	700	60.99	0.418	71.12	12.04
NP-8	800	56.93	0.386	70.34	11.92

Table-2 Optimization of pH

BATCH CODE	pH	% YIELD	DRUG LOADING (µg/mg)	ENTRAPMENT EFFICIENCY
NP-9	5.5	-	-	-
NP-10	6.5	71.2	12.04	42.93 %
NP-11	8.5	70.6	20.95	73.95 %

Table-3 Optimization of Polymer Concentration

BATCH CODE	POLYMER CONCENTRATION (mg/ml)	% YIELD	DRUG LOADING (µg/mg)	ENTRAPMENT EFFICIENCY
NP-12	20	80.0	11.07	9.74 %
NP-13	40	77.14	11.67	18.9 %
NP-14	60	71.93	27.86	62.12 %
NP-15	80	68.53	22.54	63.33 %
NP-16	100	70.6	20.95	73.95 %

Table- 4 Optimization of Amount of Cross linking agent (Glutaraldehyde)

BATCH CODE	AMOUNT OF GLUTARAL-DEHYDE (µl/mg of BSA)	% YIELD	DRUG LOADING (µg/mg)	ENTRAPMENT EFFICIENCY (EE %)	% RELEASE (after 48 hrs)
NP-17	0.6	71.22	36.63	80.87	82.13
NP-18	1.0	71.93	27.86	62.12	32.39
NP-19	1.4	71.33	22.54	48.23	31.71

Fig-1 SEM Photographs of Nanoparticles at X10000 and X30000

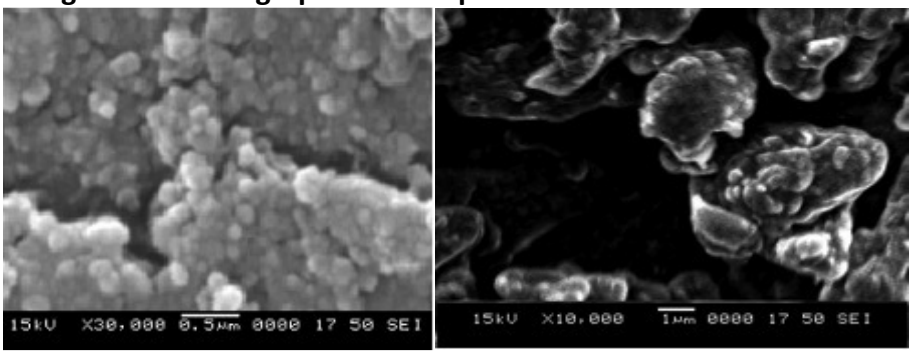


Fig- 3 FT-IR Spectra of BSAFig-4 FT-IR Spectra of nanoparticles

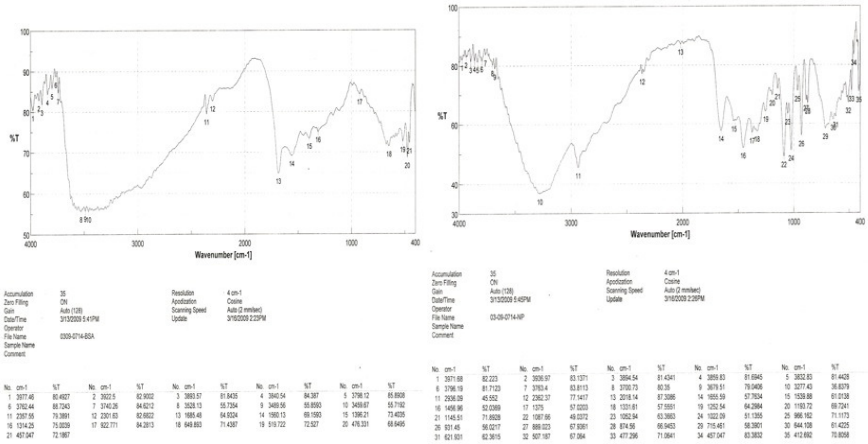
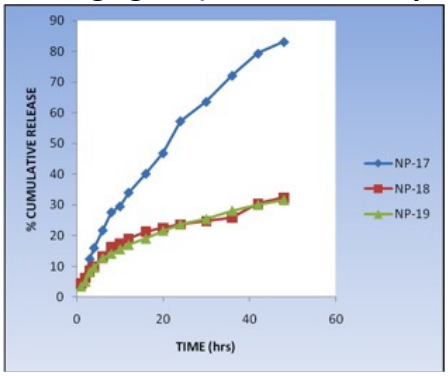


Fig-4 Effect of Amount of Cross linking agent (8% Glutaraldehyde) on % Cumulative release



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