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Case Study *Open Access*

Formulation & in vitro evaluation of amphotericin B using β-cyclodextrin capped silver nanoparticles

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ABSTRACT

The prepared polymeric silver nanoparticles so formed were evaluated for %Entrapment efficiency, and in vitro release profiles. The in vitro drug release studies were carried out with apparatus (USP II) with paddle. The formulation A7 showed a drug release of 94.68% for 30mins indicates the increased bioavailability of the drug.The formulation [A7] was selected as best formulation based on % Entrapment efficiency and drug release and was subjected to determination of particle size and zeta potential, particle morphology, in-vitro release studies. The formulation (A7] had particle size of 167.8 nm and the zeta potential (ζ) is -23.7 mV. The particle morphology of Amphotericin B loaded NPs was confirmed by Scanning Electron Microscope. Stability studies performed for optimized Amphotericin B loaded AgNPs formulations indicate that it have more stability at room temperature. The optimized formulation A7 follows Zero order kinetics with Higuchi.

Keywords: Amphotericin, B cyclodextin, Nanoparticles

INTRODUCTION

Nanotechnology is evolving rapidly with much more potential impacts in the treatment of chronic diseases such as cancer and diabetes, respiratory diseases such as asthma, and ocular diseases, and in gene therapy[1]. The development of effective drug delivery systems that can transport and deliver a drug precisely and safely to its site of action is becoming a highly important research area for pharmaceutical researchers. Indeed, a great number of new delivery technologies surface each year and nearly every part of the body has been studied as a potential route for administrating both classical and novel medicines. Nanotechnology is the creation and utilization of materials, devices and systems through the control of matter on the nanometer-length scale which is at the level of atoms, molecules and supra molecular structures. The applications of nanotechnology in various

disciplines and specifically in healthcare are becoming increasingly common and the process of replacing traditional medicines. Polymeric nanoparticles have been extensively investigated over the last two decades after the first report was published in 1976. In August 2000, the US FDA approved the first nanoparticle-mediated medicine known as Rapamune (sirolimus), an immunosuppresant to prevent organ transplant rejection. Nanoparticles are solid sub-micronic drug carriers of natural, semisynthetic, or synthetic polymeric nature in the nanometer size range. Nanoparticles may or may not be biodegradable and can be defined as solid colloidalparticles containing an active substance that are produced by mechanical or chemical means.Many methods have been developed for preparing nanoparticles; these methods can be classified into two main categories according to whether the formulation requires a polymerization reaction or is achieved directly from a macromolecule or preformed polymer. The polymerization methods can be further classified into emulsion and interfacial polymerization, and there are two types of emulsion polymerization-organic and aqueous-depending on the continuous phase.Emulsification or Solvent evaporation method: Emulsification-solvent evaporation involves two steps. The first steprequires emulsification of the polymer solution into an aqueous phase During the second step polymer solvent is evaporated, inducing polymer precipitation as nanospheres. A polymer organic solution containing the dissolved drug is dispersed into nanodroplets, using a dispersing agent and high-energy homogenization, in a non solvent or suspension medium such as chloroform or ethyl acetate.The solvent is subsequently evaporated by increasing the temperature under pressure or by continuous stirring.

Spontaneous emulsification or solvent diffusion method (ESD Technique)

It is a modified form of solvent evaporation technique. The mixture of water miscible and immiscible organic solvents are used. Due to the spontaneous diffusion of water-soluble solvent, an interfacial turbulence is created between two phase leading to the formation of smallerparticles. As the concentration of water soluble solvent increases, a considerable decrease in particle size can be achieved.

Polymeric nanoparticles (PNP)

Polymeric nanoparticles are particles of less than 1 μm diameter that are prepared from natural or synthetic polymers. The polymeric nanoparticles have become an important area of research in the field of drug delivery because they have the ability to deliver a wide range of drugs to varying areas of the body for sustained periods of time[5].

Solvent displacement and interfacial deposition

Solvent displacement and interfacial deposition are similar methods based on spontaneous emulsification of the organic internal phase containing the dissolved polymer into the aqueous external phase (Fig8). However, solvent displacement forms nanospheres or nanocapsules, whereas interfacial deposition forms only nanocapsules. Solvent displacement involves the precipitation of a preformed polymer from an organic solution and the diffusion of the organic solvent in the aqueous medium in the presence or absence of a surfactant.

Polymerization of monomers

Nanoparticles can be prepared by polymerization of monomers. The polymerization initiates by using the initiator. Initiation can be done by different types like temperature, pH, irradiation. For example,cyanoacrylic monomer is added to the surfactant solution under vigorous mechanical stirring to polymerize alkylcyanoacrylate at ambient temperature.Drug is dissolved in the polymerization medium either before the addition of the monomer or at the end of polymerization reaction

Salting out with synthetic polymers

Salting out is based on the separation of a water miscible solvent from aqueous solution via a salting-out effect. The salting-out procedure can be considered as a modification of the emulsification or solvent diffusion (Fig

10). Polymer and drug are initially dissolved in a solvent such as acetone, which is subsequently emulsified into an aqueous gel containing the salting-out agent (electrolytes, such as magnesium chloride, calcium chloride, and magnesium acetate, or non- electrolytes such as sucrose) and a colloidal stabilizer such as polyvinyl pyrrolidone or hydroxyl ethylcellulose.

Ionotropic gelation method

The ionotropic gelation method is very simple and mild. In the ionotropic gelation method polysaccharides (alginate, gellan and pectin) are dissolved in water or in weak acidic medium (chitosan). These solutions are then added dropwise under constant stirring to the solutions containing other counterions. Due to the complexation between oppositely charged species, polysaccharides undergo ionic gelation and precipitate to form spherical particles. The beads are removed by filtration, washed with distilled water and dried.

Production of Nano particles by using supercritical fluid (SCF)

Conventional methods like solvent evaporation, coacervtion and in situ polymerization often required the use of toxic solvents and surfactants. In rapid expansion of supercritical solution method the solute of interest is solubilized in supercritical fluid and the solution is expanded through a nozzle. Thus, solvent power of supercritical fluid dramatically decreases and the solute eventually precipitates. This technique is clean because the precipitated solute completely free from solvent.

Drug Introduction[18]

Amphotericin B shows a high order of in vitro activity against many species of fungi. Histoplasma capsulatum, Coccidioides immitis, Candida species, Blastomyces dermatitidis, Rhodotorula, Cryptococcus neoformans, Sporothrix schenckii, Mucor mucedo, and Aspergillus fumigatus are all inhibited by concentrations of amphotericin B ranging from 0.03 to 1.0 mcg/mL in vitro. While Candida albicans is generally quite susceptible to amphotericin B, non-albicans species may be less susceptible. Pseudallescheria boydii and Fusarium sp. are often resistant to amphotericin B. The antibiotic is without effect on bacteria, rickettsiae, and viruses.

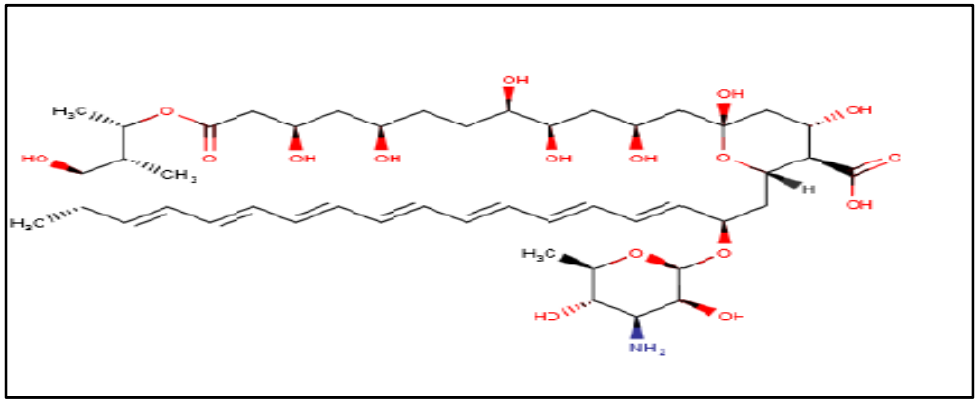

Fig 1. Amphotericin B

Amphotericin B is fungistatic or fungicidal depending on the concentration obtained in body fluids and the susceptibility of the fungus. The drug acts by binding to sterols (ergosterol) in the cell membrane of susceptible fungi. This creates a transmembrane channel, and the resultant change in membrane permeability allowing leakage of intracellular components. Ergosterol, the principal sterol in the fungal cytoplasmic membrane, is the target site of action of amphotericin B and the azoles. Amphotericin B, a polyene, binds irreversibly to ergosterol, resulting in disruption of membrane integrity and ultimately cell death.

Pre-formulation studies

Standard Calibration Curve for Amphotericin B

Table no1. Calibration curve data of Amphotericin B in 6.8 pH Phosphate buffer

S.No	Concentration	Absorbance
1	0	0
2	5	0.112±0.02
3	10	0.249±0.04
4	15	0.371±0.01
5	20	0.521±0.02
6	25	0.646±0.01

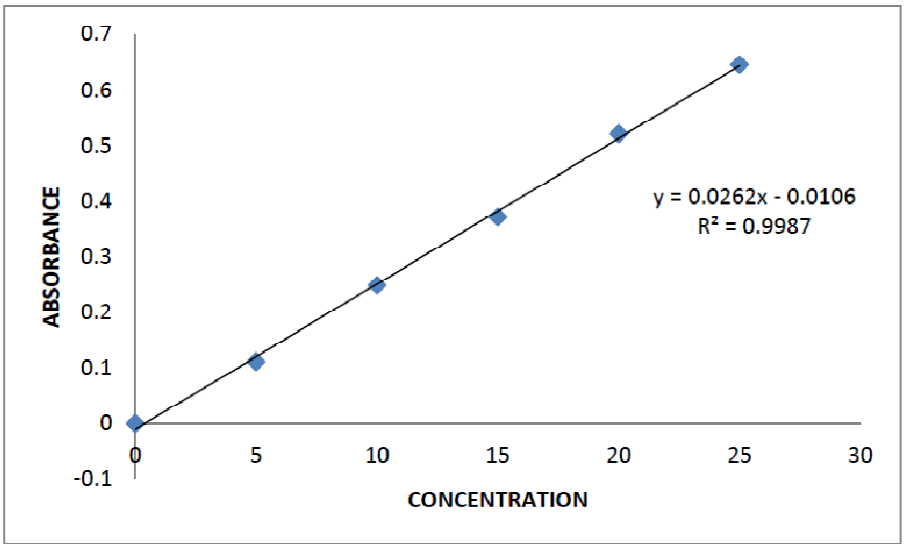

Fig 2. Calibration curve of Amphotericin B in 6.8 pH Phosphate buffer

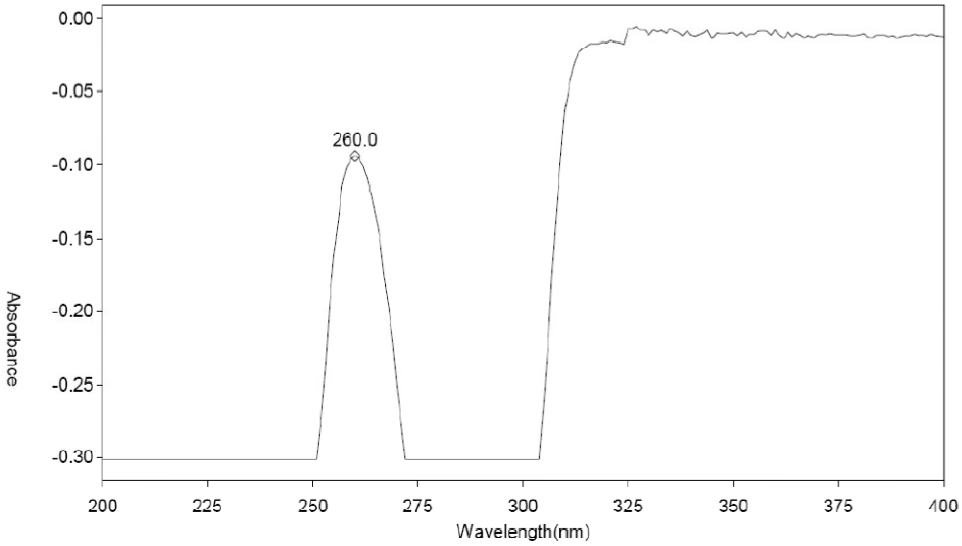

Fig no3. SPECTOPHOTOMETRY FOR Amphotericin B at 260nm

The samples were scanned at the wavelength 400-4000cm-1 by using Perkin Elmer diamond UATR model and studied for presence of characteristic peaks. The functional groups present in the pure drug Amphotericin B were observed and that helps in identification of compound. In this work the Amphotericin B API was scanned with different polymers those are Amphotericin B with AgNP solution, **β** –cyclodextrin and Amphotericin B with silver nano particles.

Evaluation of Amphotericin B using HPMC capped AgNPs

SEM

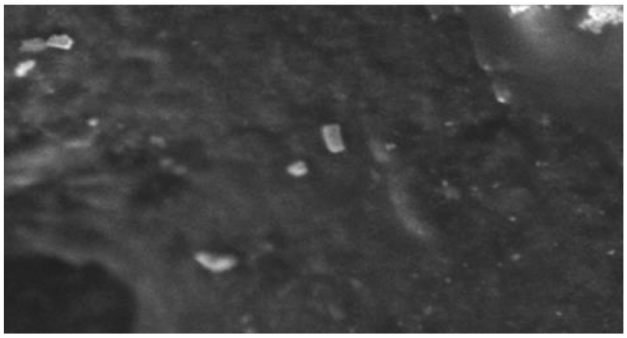

Fig 4. silvernanoparticle SEM -m01 for (A7) formulation

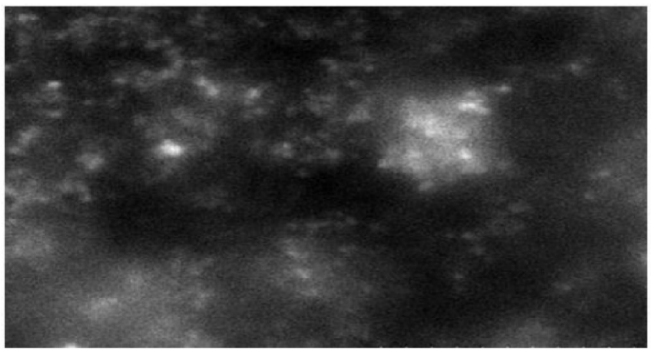

Fig 5. SEM of Ag-NP-PTX –m02 for (A7) formulation

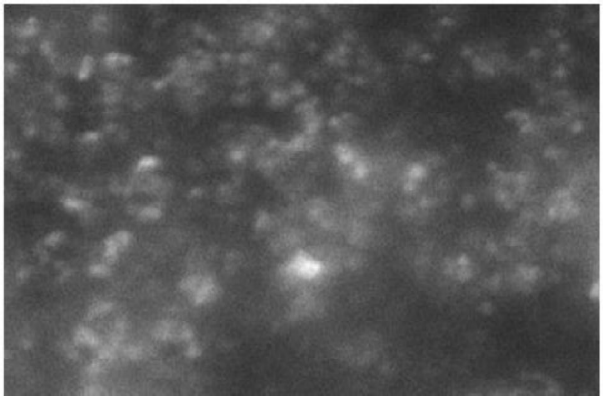

Fig 6. SEM of Ag-NP-PTX –m02 for (A7) formulation

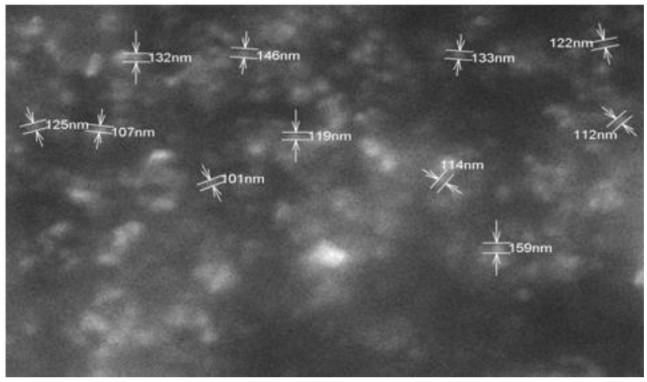

Fig 7. SEM of Ag-NP-PTX –m02s for (A7) formulation

The zeta potential of the best formulation[A7] was determined and found to be -23.7 mV. Zeta potential is an important physico-chemical parameter that influences stability of the nanosuspension. Extremely positive or negative zeta potential values cause larger repulsive forces, whereas repulsion between particles with similar electric charge prevents aggregation of the particles and thus ensures easy redispersion.

Table no 2. % Drug Entrapment Efficiency% Drug entrapped in silver nanoparticles

Formulation	% Entrapment Efficiency
A1	78.12
A2	78.46
A3	79.47
A4	81.28
A5	81.26
A6	85.51
A7	89.12
A8	85.59

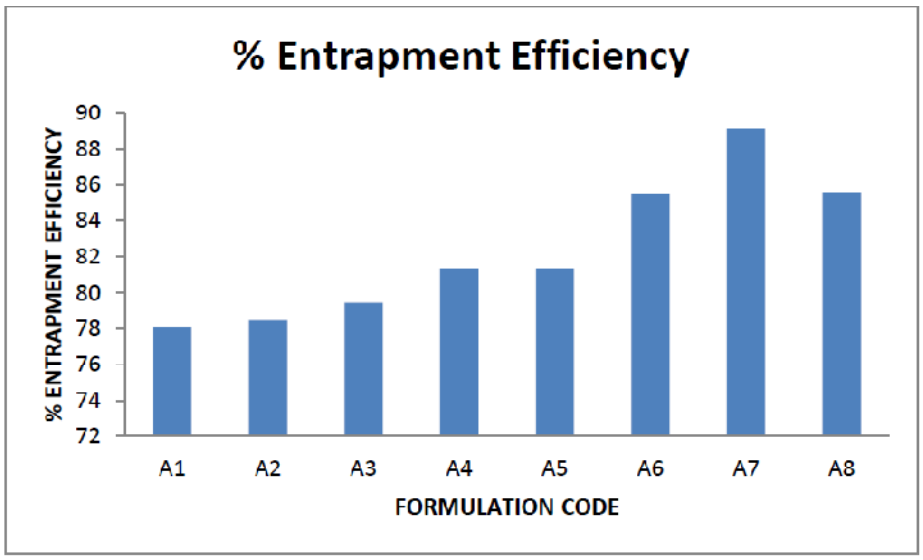

Fig 8. % Entrapment Efficiency for A1-A8

Methodology of controlled drug delivery of Amphotericin B using β-cyclodextrin capped silver nanoparticles

Method of preparation of silver nanoparticles

Nanoparticles are prepared by solvent evaporation method. Initially 5ml of deionized water and 0.0396gms of β-cyclodextrin (3.5*10-3mol) was mixed for 20min and add silver nitrate solution at different concentration and stir for 10min and add 50µL of NaOH (1M). Now place the beaker on thermostatically controlled magnetic stirrer. Initially the mixture is in white colour. Now set the temperature to 800C. heat it for 20mins.Colour changes yellow after intenfied colour is observed which indicates complete formulation of nanoparticles.

Amphotericin B loaded on silver nanoparticles

1gms of Amphotericin B was dissolved in 5ml of Ethanol and β-CD capped AgNPs was dissolved in 2ml of deionized water. The drug solution was added to β-CD capped AgNPs. This O/W emulsion was stirred overnight for removing the solvent. The Amphotericin B encapsulated β-CD capped AgNPs were harvested by centrifugation at 8000rpm for 30mins. Then washed with distilled water.

Table no 3. Formulations for Amphotericin B using β-CD capped AgNPs

Ingredients	A1	A2	A3	A4	A5	A6	A7	A8
Amphotericin B	50mg	50mg	50mg	50mg	50mg	50mg	**50mg**	50mg
β-CD	39.6	39.6	39.6	39.6	39.6	39.6	**39.6**	39.6
AgNO3	20µL (15M m)	40µL (15M m)	60µL (15M m)	80µL (15M m)	20µL (20M m)	40µL (20M m)	**60µL (20M m)**	80µL (20M m)
NaOH	50µL	50µL	50µL	50µL	50µL	50µL	**50µL**	50µL
Ethanol	5ml	5ml	5ml	5ml	5ml	5ml	**5ml**	5ml
Water	5ml	5ml	5ml	5ml	5ml	1g	**5ml**	5ml

Characterization of Silver nano particles (NPs)

Nanoparticle size distribution & zeta potential (ζ) were determined using photon correlation spectroscopy (Zetasizer, HAS 3000; Malvern Instruments, Malvern, UK). The analysis of size distribution was performed at a scattering angle of 90degrees and at a temperature of 25°C using samples appropriately diluted with filtered water by using the a disposable zeta cuvette zeta potential was measured. Molecular and crystal structures were determined by XRD analytical technique. The morphology of the microspheres was studied using scanning electron microscopy (SEM) (Malvan 4000). Microphotographs were taken on dissimilar magnification and higher magnification was used for surface morphology.

Determination of entrapment efficiency percentage

The amount of ketoprofen entrapped in prepared silver nanoparticles was estimated by centrifugation method. 1gm of silver nanoparticles was taken and diluted with 10ml phosphate buffer (pH 6.8). This suspension was sonicated using bath sonicator for 20 minutes. Later this solution was placed in centrifugation tube and centrifuged at 14000 rpm for 30 minutes. 0.5ml of supernatant was withdrawn and diluted before going for absorbance measurement using UV spectrophotometer (UV-3200 Lab India) at 235nm. This gives us the total amount of unentrapped drug. Entrapment efficiency is expressed as the percent of drug trapped.

$$\% \text{ Entrapment} = \frac{\text{Total drug} - \text{Diffused drug}}{\text{Total drug}} \times 100$$

In vitro dissolution studies

Dissolution rate of all prepared Amphotericin B using β-CD capped AgNPs formulations were performed using LAB INDIA dissolution apparatus (USP II) with paddle. The dissolution fluid was 900 ml with phosphate buffer
pH 6.8 at a speed of 50 rpm and a temperature of 37º C were used in each test. The dissolution experiments were conducted in triplicate. For all tests 5ml samples of the test medium were collected at set intervals (5, 15, 30,45, 60min) and were replaced with equal volume of phosphate buffer pH 6.8. The samples were analyzed at 235nm using a UV spectrophotometer.

In vitro drug release studies

Apparatus used : USP II dissolution test apparatus
Dissolution medium volume : 900 ml
Volume temperature : 37°C ±0.5º C
Speed of basket paddle : 50 rpm
Sampling intervals : (5, 15, 30,45, and 60min)
Sample withdrawn : 5 ml
Absorbance measured : 260nm
From the above results select the better drug release formulation then tablets were prepared with final product.

Table no4. *In-Vitro* drug release (F1to F8)

Time in mins	A1	A2	A3	A4	A5	A6	A7	A8
5	**20.48**	**16.62**	**19.21**	**22.3**	**17.42**	**19.27**	**26.42**	**22.26**
15	55.72	59.36	52.45	59.7	54.26	57.42	60.19	62.44
30	76.59	78.919	82.79	90.17	84.49	86.29	94.68	90.72

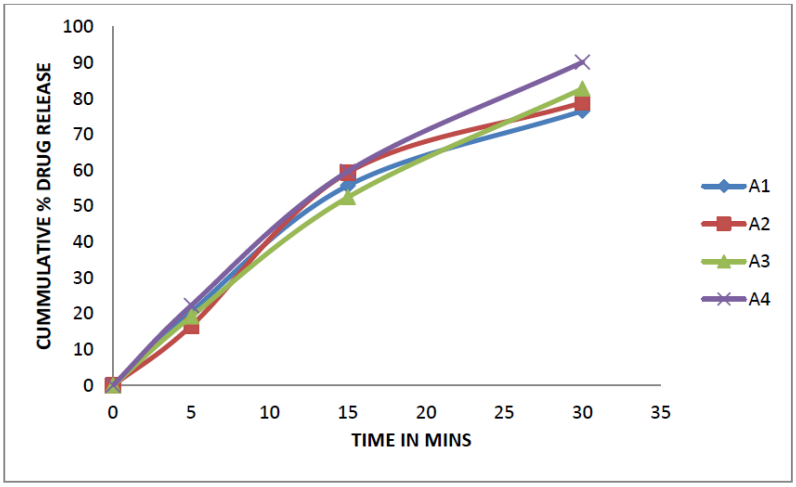

***Fig no 9. In-Vitro* drug release study for formulation A1-A4**

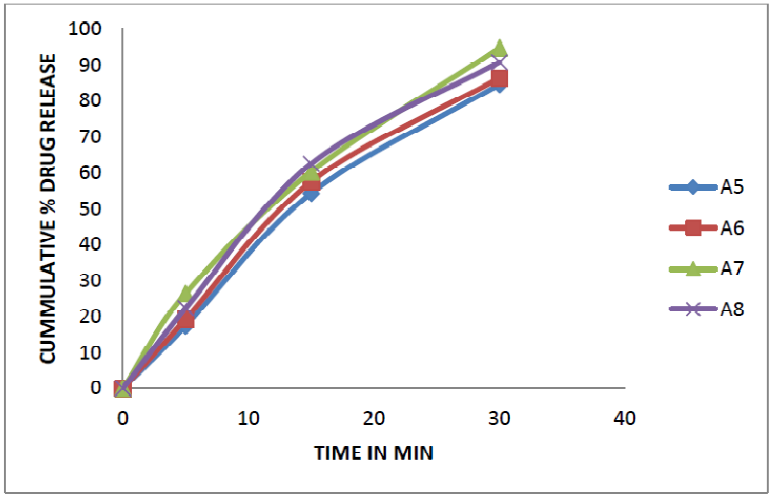

***Fig 10. In-Vitro* drug release study for formulation A5-A8**

Table no5 . In-Vitro Drug Release Kinetics R^2 values for release kinetics

RELEASE KINEITCS			
ZERO	HIGUCHI	PEPPAS	FIRST
Vs T	Q Vs √T	Log C Vs Log T	Log % Remain Vs T

Slope	3.0652	17.376	1.3395	-0.0425
Intercept	7.0076	-5.0088	0.1719	2.0791
R 2	0.9732	0.9793	0.9334	0.962

Table no6 . Stability studies% Entrapment efficiency and *invitro* drug release after stability studies(A7)

Number of Days	% Entrapment Efficiency at temperatures			% Drug release at temperatures		
	4±2°C	25±2 °C	37±2 °C	4±2 °C	25±2 °C	37±2 °C
15	89.12	89.12	89.11	94.68	94.68	94.64
30	89.12	89.10	89.04	94.22	94.0	94.52
45	88.49	88.56	88.19	93.11	93.16	93.46
90	88.14	88.14	88.07	92.00	92.08	92.12

It is clear from the results obtained that the AgNPs have shown the minimum drug lost at refrigerated condition and fairly high retention of drug was observed. At this low temperature condition % remaining drug entrapped and drug content was good over a period of months. While, storage at higher temperatures 25±2oC and 37±2oC leads to less % remaining drug entrapped and drug content over a period of 3 months respectively. So it can be inferred from the above discussion that the AgNPs formulation should be stored at lower temperature to minimize the drug loss and increase the stability of drug.

SUMMARY AND CONCLUSION

According to BCS classification, Amphotericin B comes under class IV drug i.e., low solubility and low penetrability which results in low bioavailability of drug. As extremely less particles size may enhances the solubility of drug, the objective of the present work is intended to formulate and evaluate the silver nanoparticles of Amphotericin B, in view of enhancing of drug solubility, drug bioavailability and timed release of drug molecule. The prepared polymeric silver nanoparticles so formed were evaluated for % Entrapment efficiency, and in vitro release profiles. The in vitro drug release studies were carried out with apparatus (USP II) with paddle. The formulation A7 showed a drug release of 94.68% for 30mins indicates the increased bioavailability of the drug. The formulation [A7] was selected as best formulation based on % Entrapment efficiency and drug release and was subjected to determination of particle size and zeta potential, particle morphology, in-vitro release studies. The formulation (A7] had particle size of 167.8 nm and the zeta potential (ζ) is -23.7 mV. The particle morphology of Amphotericin B loaded NPs was confirmed by Scanning Electron Microscope. Stability studies performed for optimized Amphotericin B loaded AgNPs formulations indicate that it have more stability at room temperature. The optimized formulation A7 follows Zero order kinetics with Higuchi.

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