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*Research Paper*

# Biodegradable Nanoparticles Improve Oral Bioavailability of Amphotericin B and Show Reduced Nephrotoxicity Compared to Intravenous Fungizone®

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***Purpose.*** Amphotericin B (AMB), an effective antifungal and antileishmanial agent associated with low oral bioavailability (0.3%) and severe nephrotoxicity, was entrapped into poly(lactide-co-glycolide) (PLGA) nanoparticles to improve the oral bioavailability and to minimize the adverse effects associated with it.

***Materials and Methods.*** The AMB-nanoparticles (AMB-NP) were prepared by nanoprecipitation method employing Vitamin E-TPGS as a stabilizer. *In vitro* release was carried out using membrane dialysis method. The *in vitro* hemolytic activity of AMB-NP was evaluated by incubation with red blood cells (RBCs). The acute nephrotoxicity profile and oral bioavailability of AMB-NP were evaluated in rats.

***Results.*** The prepared AMB-NP formulation contained monodispersed particles in the size range of 165.6±2.9 nm with 34.5±2.1% entrapment at 10% *w/w* initial drug loading. AMB-NP formulation showed biphasic drug release, an initial rapid release followed by a sustained release. The AMB-NP formulation exerted lower hemolysis and nephrotoxicity as compared to Fungizone®. The relative oral bioavailability of the AMB-NP was found to be ~800% as compared to Fungizone®.

***Conclusion.*** Together, these results offer a possibility of treating systemic fungal infection and leishmaniasis with oral AMB-NP, which could revolutionize the infectious disease treatment modalities.

**KEY WORDS:** amphotericin B; bioavailability; biodegradable; nephrotoxicity; oral delivery.

## INTRODUCTION

In recent decades, opportunistic fungal infections have emerged as an important causes of morbidity and mortality in immunocompromised patients, especially transplant recipients and patients with hematological malignancies, presenting major therapeutic challenges for the clinicians (1,2). AMB is one of the most effective antifungal agents used for the treatment of severe systemic fungal infections (3,4). Treatment of visceral leishmaniasis, also known as Kala-azar, is another very important therapeutic indication of AMB (5). The therapeutic arsenal against leishmaniasis is limited to a small number of parenterally administered agents, with daily injections of pentavalent antimony compound for 28 days being the usual course of treatment (6). However, due to increasing resistance, antimonial drugs can no longer be used in many areas, where the incidence of Kala-azar is highest. AMB is the current secondary treatment of choice against leishmaniasis and has a 97% cure rate with no reported resistance (7).

The unfavorable biopharmaceutical properties of AMB contribute to poor oral bioavailability limiting its oral delivery and because of these issues; it is currently administered parenterally for the treatment of systemic fungal infection or visceral leishmaniasis (8). Therapy with conventional dosage form of AMB, Fungizone®, a colloidal dispersion with sodium deoxycholate, involves intravenous administration over a period of 30 to 40 days, which is associated with infusion and drug-related side-effects (infection of the indwelling catheter, hemolysis, fever, bone pain, thrombophlebitis and most importantly, dose-dependant nephrotoxicity) (9).

Recent advances in drug delivery technology have resulted in the development of lipid-based formulations of AMB. After many attempts to decrease the toxicity of AMB by using liposomes, emulsions and other systems, three lipid formulations of AMB (Ambisome®, Amphocil®, and Abelcet®) have been developed and made commercially available. Though treatment with lipid based formulations require a shorter course of therapy (3–5 days) and are more effective and exhibit lower toxicity when compared to Fungizone®, these formulations are substantially more expensive and the higher cost of these formulations is a barrier to widespread use. Due to the difficult route of drug administration, toxicity issues and high cost, AMB is failing to reach the infected population in many regions and mortality rates continue to rise.

Nevertheless, with a background of high costs, low compliance and technical problems with administration in

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endemic countries as well as the severe toxicity, an effective oral treatment is paramount in order to address both early and late stages of this deadly disease. Oral delivery could improve patients' compliance and pharmacokinetics of a drug. A huge number of formulations have been reported to improve the delivery of AMB; however, a few formulations have been investigated for AMB oral delivery, which includes nanosuspension and lipid formulations of AMB viz., Peceol-AMB and chochleates-AMB (10–12). The nanosuspension was successful in improving the solubility of AMB, which is thought to be primary requirement for an oral formulation; however, such formulations may not offer protection from GIT degradation or prevent P-gp efflux. The nanosuspension was reported to reduce the parasite burdens by 28.6% as compared to control in the visceral leishmaniasis mice model (10). Risovic *et al*. reported a self emulsifying drug delivery system (SEDDS) using lipidic excipient Peceol for oral delivery of AMB. This formulation showed improved oral absorption of AMB; however, the huge amount of lipid (1.33 gm/kg) had to be administered, which is a large dosage volume and may cause inconvenience in administration. More recently, Cochleates containing AMB (CAMB, ~500 nm) has been developed for oral administration (12). The developed formulation demonstrated a dose dependent reduction in fungal burden in kidneys and lungs in C. albicans infected mice. The results at 2.5 mg/kg/day of oral CAMB were comparable to a deoxycholate AMB formulation administered intraperitoneally at 2 mg/kg/day. This was certainly a significant improvement considering non-availability of oral formulation for AMB; however, daily dosing would increase cost/treatment for diseases like leishmaniasis though the focus of CAMB remained to be for fungal infections. In recent years, polymeric NP are explored for oral delivery of a variety of water insoluble molecules with a potential to reduce the dose/improve oral bioavailability; thereby minimize toxicity associated with the drugs (13). These colloidal carriers have ability to cross the mucosal barrier as such and the potential for enhancing drug bioavailability via unique uptake mechanisms (14), where particle size is thought to play a crucial role (smaller the better) (15). Therefore, the present investigation is an attempt to develop AMB-NP with a view to improve oral bioavailability and minimize the adverse effects associated with AMB.

## MATERIALS AND METHODS

### Materials

PLGA (Resomer RG 50:50 H; inherent viscosity 0.41 dl/g) was purchased from Boehringer Ingelheim (Ingelheim, Germany) and AMB was gift sample from Sarabhai Chemicals (Vadodara, India). Fungizone® (Nicholas Piramal India Ltd, Mumbai, India) was purchased from a local pharmacy store. Didodecyldimethylammonium bromide (DMAB) and 1-amino-4-nitro naphthalene were purchased from Aldrich (St. Louis, MO, USA) and Sigma Aldrich Chemie (GmbH, Germany), respectively. Ethyl acetate (EA) and acetonitrile (HPLC grade) were purchased from Rankem Fine Chemicals (New Delhi, India). Vitamin E-TPGS was a gift sample from Eastman Chemical Company (UK). Dimethyl sulfoxide (DMSO) was LR grade and purchased from Central Drug House (P) Ltd. (New Delhi, India). Ultrapure water (SG Water Purification System, Barsbuttel, Germany) was used for all the experiments.

### Preparation of NP

NP were prepared by emulsion–diffusion–evaporation and nanoprecipitation methods. In emulsion–diffusion–evaporation method, 5 mg AMB was dissolved in 1 mL of DMSO to which 2 mL of PLGA solution (2.5% *w*/*v* in EA) was added with stirring. Organic phase was then added, drop by drop to 5 mL of Vit. E-TPGS solution (1.4% *w*/*v*) with stirring at 1,000 rpm to get primary emulsion, which was homogenized at 15,000 rpm for 5 min and diluted with water up to 25 mL. EA was evaporated under vacuum and the particles were subsequently washed with water to remove DMSO and the unbound drug as well as stabilizer.

In nanoprecipitation method, 5 mg AMB was dissolved in 0.65 mL of DMSO and then 2 mL PLGA solution (2.5% *w*/*v* in acetone) was added to it with stirring. Organic phase was then added drop by drop to the 5 mL of 50% ethanol solution containing 1.4% *w*/*v* of Vitamin E-TPGS, which was stirred at 1,200 rpm to form the NP. This NP suspension was then diluted up to 25 mL with water and organic solvents were removed under vacuum. NP were washed with water to remove DMSO, the unbound drug and stabilizer. The prepared NP formulations were characterized for particle size, polydispersity index (PDI), zeta potential and morphology.

### Characterization of the Particles

The size and PDI of the NP were determined by Dynamic Light Scattering (Nano ZS, Malvern Instruments, UK) and were taken as an average of five measurements. The surface charge of the NP was characterized in terms of zeta potential, which was determined by measuring electrophoretic mobility under an electric field as an average of 30 measurements. The amount of the drug entrapped in the NP was determined by the validated reverse phase high performance liquid chromatographic (RP-HPLC) method. Fixed volume of the NP formulation was centrifuged at 30,000 rcf for 30 min and the pellet obtained was dissolved in DMSO, diluted with acetonitrile and analyzed by validated HPLC.

The morphology of blank and drug loaded NP was analyzed by atomic force microscope (AFM) (Veeco Bioscope II) attached with Nikone eclipse TE 2000-S microscope configured to nanoscope software. The NP suspension were placed on a silicon wafer with the help of a pipette and allowed to dry in air. The microscope was vibration damped and measurements were made using commercial pyramidal Si3N4 tips (Veeco, USA). The cantilever used for scanning was 325×26 μm with a nominal force constant of 0.1 N/m. The scan size was kept 2×2 μm with scan rate of 0.996 Hz for all the NP and the images were obtained in contact mode. Images were obtained by displaying the amplitude signal of the cantilever in the trace direction, and the height signal in the retrace direction, both signals being simultaneously recorded.

### Initial Drug Loading Study

Pay load to the NP was optimized by conducting initial drug loading study. AMB at 5, 10 and 15% *w/w* of polymer was used for the preparation of the NP and its effect on particle size, PDI and entrapment efficiency was evaluated.

### *In Vitro* Drug Release Study

The *in vitro* release of AMB from the NP was carried out by dialysis membrane method. AMB-NP equivalent to 1 mg of entrapped drug were dispersed in 0.5 mL of release medium (consisting of 10 mM HEPES buffer containing 0.25% *w/v* of sodium lauryl sulphate (SLS) in dialysis bags (Sigma) with a molecular cut-off of 12,000 Da. The dialysis bags were suspended in screw capped tubes containing 10 mL of release media and incubated at 37°C in an orbital shaking water bath (80 rpm). At specified time intervals (2, 6, 12, 24 h on first day followed by sampling at 24 h interval for subsequent days), the release medium was completely replaced with fresh buffer and the amount of AMB released in medium was estimated by validated RP-HPLC method.

The cumulative amount of AMB released from the NP was calculated using the following equation.

$$\%\ Cumulative\ release\ =\ M_t/M_0 \times 100$$

Where, $M_t$ is the amount of AMB released from the NP at time ($t$) and $M_0$ is the initial amount of AMB present in the NP. The cumulative % of AMB released was plotted *versus* time.

### *In Vitro* Hemolysis Study

Fresh heparinized blood obtained from Sprague Dawley (SD) rats was used to evaluate the *vitro* hemocompatibility of the AMB-NP. The red blood cells (RBCs) were separated by centrifuging the whole blood at 3,000 rcf for 15 min. The supernatant was discarded and the RBCs were washed thrice with normal saline (NS). The stock of RBCs was prepared by mixing three volumes of the centrifuged RBCs with 11 parts of the NS and 100 μL of this stock was used for 1 ml of the sample. RBCs were mixed with distilled water (positive control); with normal saline (NS) (negative control); 2 and 5 μg/mL of AMB (Fungizone®) in NS; blank NP equivalent to 5 μg/mL of drug-loaded NP in NS; 2 and 5 μg/mL of AMB-NP in NS. Samples were incubated at 37°C for 12 h in a water bath. At predetermined time intervals (30 min, 6 and 12 h), the samples were withdrawn and centrifuged at 3,000 rcf for 15 min to separate intact RBCs. The supernatant was collected and kept for 30 min at room temperature to oxidize hemoglobin (Hb) and then the absorbance of Oxy-Hb was measured spectrophotometrically at 540 nm. The percentage of RBC lysis was calculated based on the assumption that 100% RBC lysis resulted from mixing of RBCs with distilled water.

### Nephrotoxicity Study

Nephrotoxicity studies were performed on male SD rats weighing 200–240 g. The animals were housed under standard conditions of temperature (22±2°C), light and dark cycle (12/12 h). The animals were kept on standard pellet diet and water was made available *ad libitum*. The experimental protocols were approved by the institutional ethical committee of NIPER, India. The animals were randomly divided into six groups. Group-I was kept as control without any treatment; Group-II was treated with Fungizone® at the dose of 5 mg/kg body weight as slow intravenous (i.v.) injection; Group-III received i.v. blank NP equivalent to that of AMB-NP; Group-IV was administered AMB-NP i.v. equivalent to 10 mg/kg of AMB; Group-V was administered blank NP equivalent to 10 mg/kg AMB-NP orally and Group VI was treated with AMB-NP orally equivalent to 10 mg/kg AMB. The animals were fasted for 6 h in case of oral treatments. Following 72 h post-dose, blood samples were withdrawn from the retro-orbital plexus and plasma was separated by centrifugation at 3,000 rcf for 15 min. The plasma samples were analyzed for biochemical parameters including blood urea nitrogen (BUN) and plasma creatinine (PC). BUN and PC were measured colorimetrically using commercially available kits (Accurex Biomedical Pvt. Ltd, Mumbai, India).

### Bioavailability Study

The pharmacokinetics of AMB-NP was assessed in male SD rats weighing between 200–240 g. AMB-NP (equivalent to a dose of 10 mg/kg of AMB) were dispersed in 1 ml of water and then administered orally using oral gavage needle. The relative bioavailability of the nanoparticulate formulations was assessed by administering the commercial formulation (Fungizone®) orally. After administration, blood samples were collected (500 μl) from the retro-orbital plexus under mild ether anesthesia at 0.5, 1, 2, 6, 12 and then every 24 h for 6 days in the heparinized micro-centrifuge tubes (20 units of heparin/ml of blood). Blood samples were centrifuged at 3,000 rcf for separation of plasma. The plasma samples were again centrifuged at 30,000 rcf for 30 min to avoid chances of error due to presence of intact NP present in systemic circulation. AMB concentration in plasma was determined by validated HPLC method using 1-amino-4-nitro naphthalene as an internal standard (IS). Briefly, 50 μL of internal standard solutions (2.5 μg/ml) in methanol were spiked to plasma samples (250 μL) and vortexed for 5 min for uniform mixing. To this, 1 mL of methanol was added to

**Table I.** The Characteristics of the Particles Made by Two Methods with 10% Initial Drug Loading

| Method | Particle size (nm) | PDI | Zeta potential (mV) |
|---|---|---|---|
| Emulsion–diffusion–evaporation method | 182.6±6.0 | 0.33±0.03 | −16.1 |
| Nanoprecipitation method | 165.6±2.8* | 0.18±0.01* | −15.4 |

Nanoprecipitation method *vs* emulsion–diffusion–evaporation method
*$p<0.05$

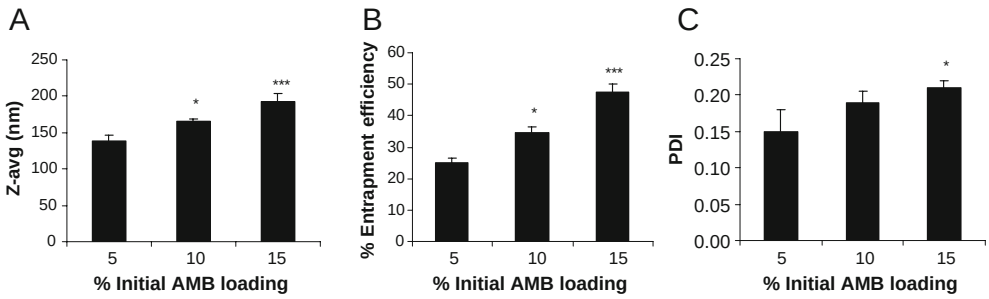


**Fig. 1.** Effect of initial drug loading on (**A**) particle size, *$p<0.05$, 5 *vs.* 10; ***$p<0.001$, 5 *vs.* 15, (**B**) entrapment efficiency, *$p<0.05$, 5 *vs.* 10; ***$p<0.001$, 5 *vs.* 15, and (**C**) PDI, *$p<0.05$, 5 *vs.* 15 (data represented as mean ± SD, $n=3$).

precipitate out the plasma proteins, which were subsequently removed by centrifugation at 20,000 rcf for 10 min. The supernatants were collected and vacuum-dried. Residues left after drying were reconstituted in 75 μL methanol, centrifuged again at 20,000 rcf for 10 min and 50 μL of supernatant was injected into HPLC. All separations were carried out on the Nucleosil® 1005 $C_{18}$ columns and detection was carried out at 407 nm.

### Statistics

Statistical difference between groups was evaluated using one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison test using SigmaStat 2.03 software and a value of $p<0.05$ was considered statistically significant.

## RESULTS AND DISCUSSION

### Preparation of NP

A particular method of NP preparation is usually employed considering the physicochemical properties of the drug to be entrapped. For hydrophobic drugs, nanoprecipitation (16) or emulsion–diffusion–evaporation method is best suited while double emulsion method suits for hydrophilic drugs, such as proteins and peptides (17). In nanoprecipitation method, a single step is involved in the formation of NP. On addition of organic phase containing drug and polymer into aqueous phase, desolvation of polymer occurs rapidly due to diffusion of solvent into aqueous phase. This results into precipitation of polymer along with drug entrapped into the polymer matrix to give instantaneous formation of NP in which drug is distributed into the polymer matrix. In emulsion–diffusion–evaporation method, two steps are involved, first formation of emulsion with small droplet of organic phase containing drug and polymer in aqueous phase, then homogenization or probe sonication to reduce the globule size and subsequent removal of organic solvent by diffusion and evaporation results in formation of NP. NP prepared by both the methods were characterized in terms of size, PDI and zeta potential (Table I).

Various process parameters can influence the particle size and characteristic of final formulation. Recently, we have found that viscosity of the organic phase can be very influential on the particle size (18). For 10% *w*/*w* of initial loading, 1 mL of DMSO (viscous in nature) is required in combination with EA for solubilization of the drug in case of the emulsion–diffusion–evaporation method while nanoprecipitation method required only 0.65 mL of DMSO in combination with acetone to solubilize the same amount of drug. The higher amount of DMSO in the organic phase could lead to increase in the viscosity of the organic phase resulting in significantly ($p<0.05$) higher particle size. In nanoprecipitation method, instant diffusion of organic solvent results in formation of NP while the diffusion is slow in case of emulsion–diffusion–evaporation method.

Particle size is an important parameter, which can influence drug release, intestinal uptake and thus overall bioavailability of the formulation (15). Smaller sized particles are reported to be absorbed more efficiently as compared to larger sized particles; in contrast larger particles might sediment quickly on storage, though re-suspension could be easier, when compared to smaller ones. The zeta potential value is another important particle characteristic, which can influence particle stability as well as mucoadhesivity and thus bioavailability. More pronounced values of zeta potential,

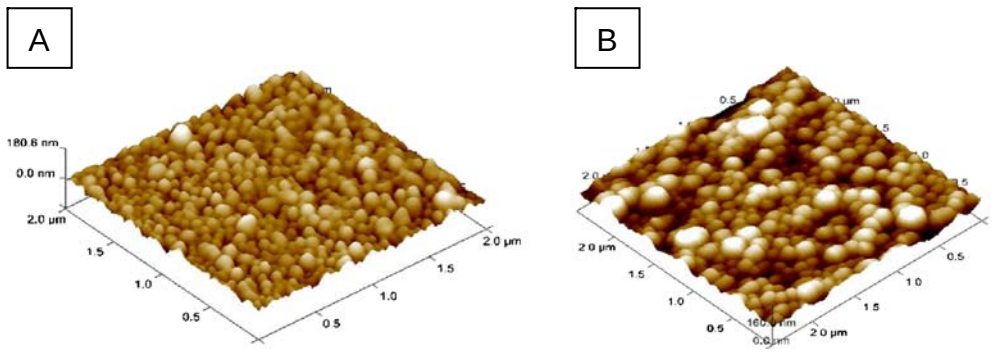


**Fig. 2.** AFM pictures of blank (**A**) and AMB-NP (**B**).

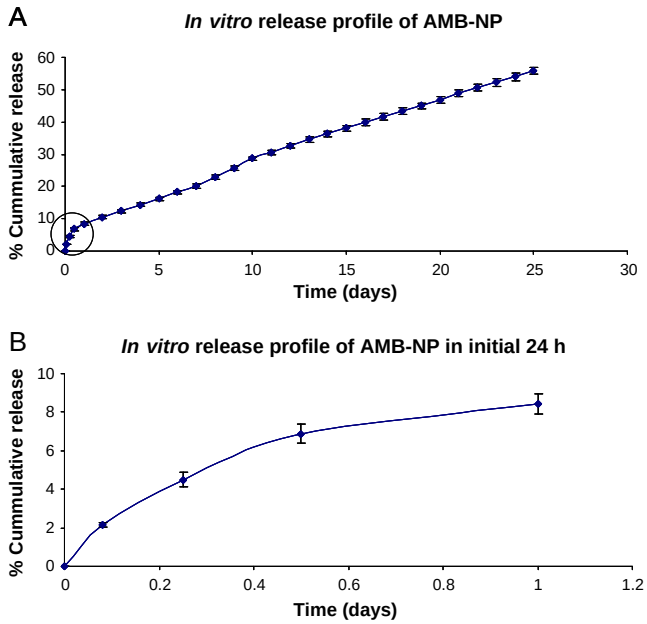


**Fig. 3.** *In vitro* release profile of AMB-NP (**A**); encircled part (**B**) indicates rapid release in the initial 24 h (data represented as mean ± SD, $n=6$).

either positive or negative, tend to stabilize the particle suspension to a greater extent. The particles with like charges tend to aggregate to lower extent due to electrostatic repulsion. The entrapment efficiency can also influence the overall bioavailability, as entrapment efficiency increases; increased amount of drug goes into the system per particle absorbed and can also result in reduced dosing requirements. However, the role of polydispersity along with one of the above discussed parameters is difficult to predict. The process optimization was aimed at producing monodispersed and small particles with highest entrapment efficiency and stabilized by surfactant coating to facilitate GI uptake. Choice of nanoprecipitation method over emulsion–diffusion–evaporation method was justified based on the fact that this method provided smaller particle size and low PDI of AMB-NP (Table I).

In initial drug loading study, percentage drug loading was increased to investigate its effect on particle size, size distribution and entrapment efficiency. A significantly ($*p<0.05$, $***p<0.001$) higher particle size was observed when particles were prepared with 10% and 15% *w*/*w* initial drug loading, respectively as compared to 5% *w*/*w* drug loading (Fig. 1A). Similarly, particles prepared with 10% and 15% *w*/*w* drug loading showed significantly ($*p<0.05$, $***p<0.001$) higher entrapment efficiency as compared to particles made with 5% initial loading (Fig. 1B). Upon increase in initial loading from 5% to 10% *w*/*w*, no significant difference in PDI values was observed; however, particles made with 15% *w*/*w* of initial loading showed significantly ($*p<0.05$) higher PDI values as compared to particles made with 5% *w*/*w* initial loading (Fig. 1C). The reason for the increase in particle size could be due to the increase in the requirement of DMSO from 0.45 to 0.85 mL with increase in the drug loading from 5% to 15% *w*/*w* of polymer. As DMSO is viscous in nature, use of higher amount of DMSO in particle preparation could lead to increase in the over all viscosity of the organic phase and thus particle size. AFM image of blank and AMB-NP prepared by nanoprecipitation method (10% *w*/*w* initial drug loading) showed smooth and spherical appearance (Fig. 2) and size measured by AFM correlated with that of zeta sizer.

## *In Vitro* Drug Release Study

The maintenance of sink conditions for formulations containing poorly soluble drugs is difficult. Since AMB has poor aqueous solubility, 0.25% *w*/*v* of SLS was added to the release medium to maintain the sink conditions. *In vitro* release profile of AMB-NP is shown in Fig. 3. Release profile indicated biphasic release of AMB from the nanoparticulate formulation. In first phase, (Fig. 3B), there was an initial rapid release of about 10% of drug in first 24 h followed by ~60% of the drug release in 25 days (Fig. 3A). During formation of NP some drug tends to migrate towards the outer surface of particles and exhibits rapid release effect (19). The release mechanism can be best described by diffusion alone during the initial phase followed by combined diffusion and degradation in the later phases.

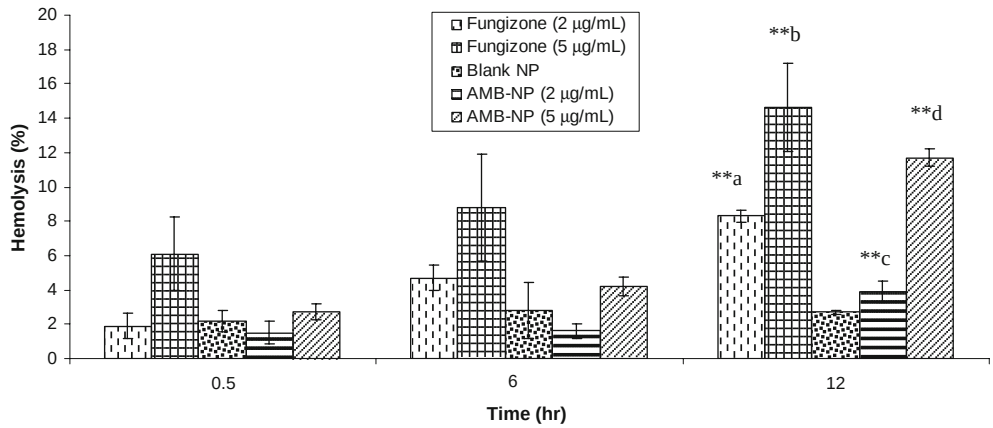


**Fig. 4.** *In vitro* RBC lysis following incubation of RBCs with Fungizone® at concentration of 2 and 5 µg/ml in (normal saline); blank nanoparticles equivalent to AMB nanoparticles (5 µg/ml) in NS; AMB nanoparticles at a concentration of 2 and 5 µg/ml in NS (**$P<0.01$, *a vs. c* and *b vs. d*, all values reported are mean ± S.D., $n=3$).

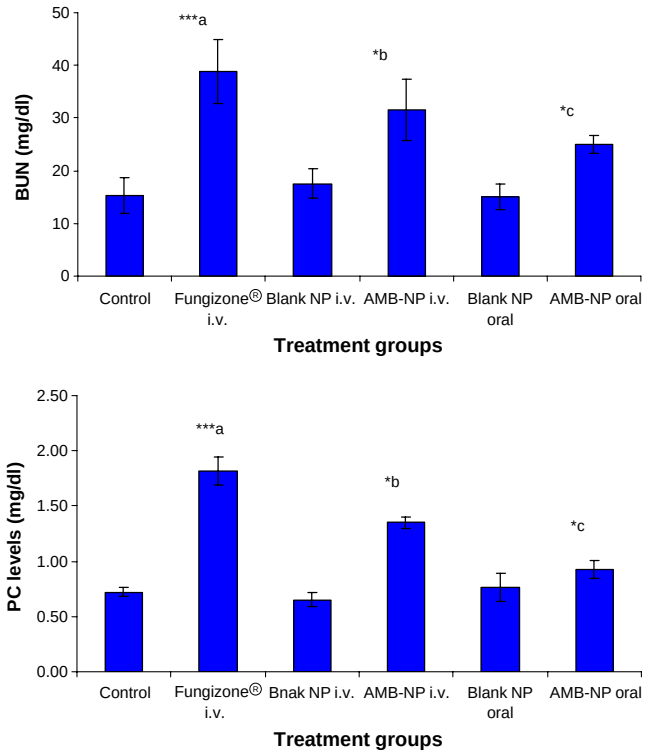


**Fig. 5.** Blood urea nitrogen (BUN) and plasma creatinine (PC) in rats on 3rd day of the study, ***$p<0.001$, *a vs.* control, *$p<0.05$, *b vs.* Fungizone® and *c vs.* control, data represented as mean ± SD, $n=3$.

The percent cumulative release was fitted into different release models viz. zero order, first order, Higuchi's square root plot and Hixson Crowell cube root plot. Among all release models, one with correlation coefficient close to unity was preferably chosen to designate the order of release. The release pattern was found to be best fitted in first order release kinetic model with $r^2$ of 0.991, which indicates that release of drug from the polymer matrix was dependent on the amount of AMB present in the matrix (20).

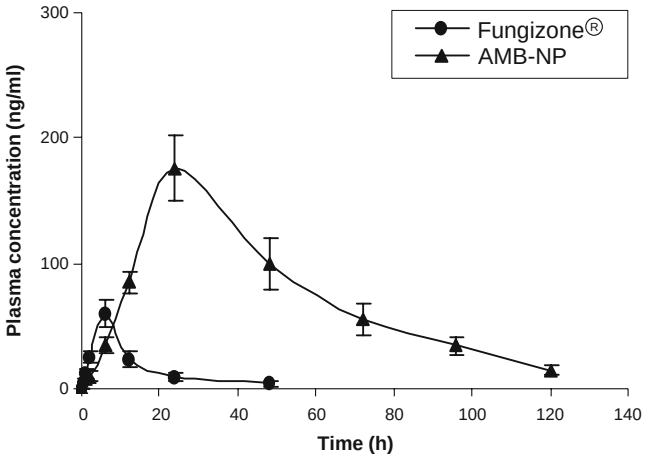


**Fig. 6.** Comparative *in vivo* plasma concentration *vs.* time profiles of AMB (10 mg/kg body weight of animal as a single dose) administered orally as Fungizone® and AMB-NP (all values reported are mean ± SD, $n=3$).

**Table II.** Pharmacokinetic Parameters of AMB Upon Oral Administration of Fungizone® and AMB Nanoparticles

| Formulation type | $C_{max}$ (ng/mL) | $T_{max}$ (h) | $AUC_{0-inf}$ (ng h/mL) |
|---|---|---|---|
| Fungizone® | 60.2±10.7 | 6 | 1,000.9±159.0 |
| AMB nanoparticles | 176.0±25.8 | 24 | 7,938.8±1,450.7 |

## *In Vitro* Hemolysis Study

In order to determine the effect of the nanoencapsulation of AMB on hemolytic profile, the RBS lysis induced by AMB-NP was compared with the Fungizone®. The extent of hemolysis induced after incubation of RBCs with different formulations of AMB is depicted in Fig. 4. Incubation of RBCs with distilled water (as positive control) led to instant RBC lysis and was considered to cause 100% hemolysis; addition of distilled water exerts osmotic pressure in RBCs resulting into rupture and hemolysis. As a negative control, normal saline exerts equal osmotic pressure across the cell membrane of RBCs maintaining the integrity of cell membrane with no hemolysis. Incubation with 2 and 5 μg/ml of Fungizone® (commercial formulation) led to significant RBC lysis compared to NS (negative control) after 12 h. However, the RBCs lysis in the presence of 2 and 5 μg/ml of AMB-NP was significantly (**$p<0.01$) lower as compared to the respective concentrations of Fungizone® (Fig. 4). In contrast, no significant Hb release was observed with blank NP after 12 h incubation with RBCs, which was comparable to the percentage of RBC lysed in the normal saline group suggesting that NP as such do not cause lysis.

The slow and sustained release of AMB from AMB-NP showed low extent of hemolysis in comparison to Fungizone®. Reason for more extent of hemolysis caused by Fungizone® could be explained based on the fact that this formulation is micellar dispersion of AMB with sodium-deoxycholate. Sodium deoxycholate is a surfactant and can induce hemolysis itself in addition to the hemolysis caused by AMB. The data therefore, indicates the protective role of NP in preventing the RBC lysis.

## Nephrotoxicity Study

AMB has wide therapeutic applications in an array of severe systemic fungal infections and leishmaniasis. However, its clinical use is limited by severe adverse effects, the most important of which is nephrotoxicity. AMB nephrotoxicity is manifested by renal insufficiency due to glomerular and vascular disease in addition to the abnormalities in tubular function. Acute AMB-induced nephrotoxicity is characterized by increased BUN, PC levels. BUN is an important biochemical marker, which is routinely evaluated as an indicator of clinical renal function. Abnormal rise in the BUN levels indicates the dysfunction or damage to the kidney. Similarly, PC is another reliable marker of the clinical renal function. Creatinine, the end product of muscle metabolism, is also filtered and excreted by the kidneys. The elevated PC levels are also important indicative of renal dysfunction.

The present investigation was conducted to determine the *in vivo* nephrotoxic potential of the AMB-NP. In the rats

treated with i.v. Fungizone®, there was a significant (***$P<0.001$) increase in BUN and PC levels as compared to control rats, indicating nephrotoxicity in the group. However, i.v. as well as oral administration of AMB-NP led to significantly (*$P<0.05$) lower BUN and PC levels in the rats, indicating lower nephrotoxicity by AMB-NP as compared to the Fungizone® (Fig. 5). On the other hand, blank NP administered i.v. and oral showed no abnormal rise in BUN or PC levels indicating the nephrotoxicity caused was due to the encapsulated AMB.

The reduced toxicity of AMB-NP observed could be due to the gradual release of the incorporated AMB dose from the NP formulation, or as a result of reduced exposure as the AMB-NP are sequestrated in tissues. Fungizone® is micellar formulation of sodium-deoxycholate containing AMB, which rapidly releases drug following its dilution in bloodstream upon its i.v. administration leading to higher plasma levels of AMB and subsequent higher nephrotoxicity.

### Bioavailability Study

The AMB-NP were designed to improve the oral bioavailability of the drug. Blood levels of AMB after oral administration of AMB-NP and Fungizone® were determined using HPLC. The mean AMB concentration in the plasma following a single oral dose of AMB-NP and Fungizone® at 10 mg/kg in rats is illustrated in Fig. 6. The relevant pharmacokinetic parameters including $C_{max}$, $T_{max}$ and $AUC_{0-inf}$ are listed in Table II.

The concentration–time profile of AMB-NP was quite different from that of oral Fungizone®. It was observed that the nanoparticulate formulation showed sustained release of AMB over a period of 5 days as compared to Fungizone®, which showed detectable AMB release for a period of only 2 days. Fungizone® upon oral administration led to very low plasma concentration with $C_{max}$ of 60.2±10.7 ng/mL and $T_{max}$ of 6 h followed by rapid decline in the plasma concentration. Whereas, relatively slow increase and sustained plasma concentration of AMB was observed for a longer time after administration of AMB-NP formulation, with significantly delayed $C_{max}$ of 176.0±25.8 ng/ml occurring at 24 h.

The relative bioavailability of AMB was 793.2% for the NP formulation as compared to Fungizone®. The bioavailability enhancing potential of the nanoparticulate formulation has been already proven for certain poorly bioavailable drugs like cyclosporine A and estradiol (21,22). When AMB is administered in free form (as present in the Fungizone®), it is absorbed by passive diffusion through intestinal membrane. However, the process of passive diffusion is dependant on the molecular weight of the drug. The passive diffusion process is efficient for the drugs up to the molecular weight of 500 Da and the efficiency decreases with increase in molecular weight of the drug molecule. Accordingly, AMB has molecular weight of 924.08 Da and thus its passive diffusion should be very low. AMB is relatively unstable at acidic pH of gastrointestinal tract and could have been degraded in the GIT prior to its absorption upon its oral administration as Fungizone®. In addition, it also undergoes extensive P-gp efflux from the enterocytes, which further limits it oral bioavailability. Together, these factors lead to very low oral bioavailability of AMB (~0.3% of the administered dose) (23). On the other hand, NP can protect the entrapped drug molecules from the enzymatic and chemical degradation in the GIT. These polymeric nanocarriers could also provide the protection against the P-gp efflux from the enterocytes. In addition, these colloidal carriers have the ability to cross the mucosal barrier as such by potential endocytic uptake mechanisms thus could enhance the lymphatic transport of AMB in the particulate form. All these factors together could be accountable for the improved bioavailability of the AMB-NP formulation.

## CONCLUSION

The present study describes a new and efficient method of making AMB encapsulating PLGA NP for oral administration, employing DMSO as a co-solvent. The developed AMB-NP demonstrates the proof of concept of improved oral bioavailability. Further, the promise of these formulations is reduced nephrotoxicity as compared to Fungizone® upon i.v. and oral administration.

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