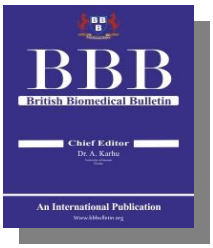
Journal Home Page www.bbbulletin.org

BRITISH BIOMEDICAL BULLETIN



Original

An *Ex vivo* study of Amphotericin-B Nanoparticle for Ocular Delivery

Swarnali Das Paul*[1], Rupa Mazumder[2], Snehendu Bhattacharya[3], and Arvind Kumar Jha.[4]

[1]*Department of Pharmacy, Uttarakhand Technical University, Dehradun, Uttarakhand, India*
[2]*NIET, Noida, India*
[3]*Shambhunath Institute of Pharmacy, Allahabad, India*
[4]*Shri Shankaracharya Group of Institutions, Faculty of Pharmaceutical Sciences, Bhilai, Chhattisgarh, India*

ARTICLE INFO

Received 19 July 2013
Received in revised form 24 July 2013
Accepted 10 Aug 2013

Keywords:
Amphotericin-B,
Goat eye,
Nanoparticle,
Ocular.

Corresponding author: Swarnali DasPaul, Assistant Professor SSIPS, Junwani, Bhilai, C.G., India.
E-mail address:
swarnali4u@rediffmail.com

ABSTRACT

Our aim was to entrap Amphotericin-B, within the nanoparticles so that it could penetrate the cornea effectively. Solvent displacement method was used to prepare the nanoparticles. Confocal laser scanning microscopy was used to check the corneal penetration of the nanoparticle into isolated goat eyes after contact time of 1 hr and 24 hrs with the formulations. Prepared nanoparticles had particle size range from 112 to 350 nm with a zeta potential of 19 to 34 mV. This positive zeta helps the nanoparticle to adhere with corneal mucosa and release the drug. Almost 60-70% of the drug was released from every formulation in 6 to 7 hrs. The nanoparticles were able to entrap upto 75% of the drug successfully. Ex-vivo study reveals that 50% of the topically applied drug penetrated the cornea within 24 hrs whereas the plain drug solution had negligible penetration capacity.

Introduction

Ocular infections like keratitis, orbital cellulites, endophthalmitis and dacryocystitis can lead to severe damage in ocular tissues. Fungal Keratitis is one of the dangerous disease which leads to blindness. In India, nearly 30-35% of all culture positive infectious keratitis are caused by fungi[1]. Treatment of fungal infection is difficult. The antifungal agents available today to combat fungal keratitis are not so well developed as those available against bacterial infections. Most antifungal medications are merely fungistatic, and require an intact immune system and prolonged therapeutic course. Except for Natacyn (natamycin 5%, Alcon), all antifungal medications are chosen from systemic drugs[2]. Amphotericin-B (AmB) is such a drug of choice. Natacyn and AmB both belong to polyene antibiotic. Polyenes disrupt the cell by binding to fungal cell wall ergosterol and are effective against both filamentous and yeast forms.

Amphotericin-B, a polyene antibiotic with broad spectrum antifungal activity is a choice of severe ophthalmic fungal infections like fungal keratitis. But no ophthalmic formulation is available of this large ploypeptide so development of an ophthalmic formulation would be beneficial as well as economical. The macrostructure of AmB, hinders it from successful corneal penetration[3]. Nanoparticles are reported to deliver large molecules including polypeptides successfully after topical application.

Nanoparticles are suitable and promising drug delivery systems for ophthalmic applications mainly for two reasons. First, nanoparticles are efficient to bind with ocular mucosa and release the drug over there for a prolong time so drug absorption in the eye is enhanced significantly in comparison to eye drop solutions. Hence it slower ocular elimination rate of particles. Second, eye better tolerate the smaller size of nanoparticles so patient comfort is also achieved. Several researchers have reported the efficacy of nanoparticles to deliver the drug in ocular tissue after topical application[4-6]. Here we tried to deliver the AmB nanoparticles in goat cornea after topical application.

Today all the experimental results needs to confirm through animal study. The living animal suffers a lot of pain and stress during these experiments. A large no. of living animals is used every time for each of the experiments. Due to the inhuman use of these living animals it is our moral duty to substitute these experiments protocol with isolated animal body part. For this reason we performed *Ex vivo* study on isolated goat eye's before performing experiments with living rabbit's eyes. So, the permeation and retention of the nanoparticle suspension into the corneal cells was evaluated using an *Ex vivo* (excised goat cornea) approach.

Materials and Methods

Materials

Amphotericin-B was obtained from Synbiotics Pvt. Ltd., Vadodara, India. Eudragit® RS100 and Eudragit® RL100 were obtained from FDC Ltd., Mumbai. Acetone, Polyvinyl alcohol (PVA), dimethyl sulfoxide (DMSO) and methanol were purchased from S. D. Fine Chemical Limited, Mumbai.

Preparation of Nanoparticle

The polymers, Eudragit® RS100 or Eudragit® RL100 and drug, AmB were first dissolved, by heating and sonication, in 20 ml of acetone and methanol (3:1). This organic phase was adjusted to pH 4 with HC1 in order to promote AmB solubilization. This solution was injected into an aqueous solution containing a hydrophilic surfactant under moderate magnetic stirring. Finally, the organic solvents were evaporated under

reduced pressure at 58°C. Various process variables, which could affect the preparation and properties of the nanoparticles, were studied like effect of polymer weight, stirring effect during manufacturing, volume of aqueous phase etc.

In vitro Characterization

The nanoparticles were evaluated by particle size and zeta potential by Photon Correlation Spectroscopy (PCS) with a Malvern Zetasizer Nano ZS (Malvern Instrument Ltd., UK). The morphological examination of the Nps was performed with a transmission electron microscope (TEM; CM12 Philips, USA). AmB release from Nanoparticle was evaluated using diffusion cells in simulated tear fluid of pH 7.4. % drug entrapment efficiency and yield of nanoparticle were also determined.

Ex vivo corneal retention and penetration studies

Goat cornea was excised immediately after the animal sacrifice (local slaughter-house, Bhilai, C.G.) and put in an iced (4 °C) Krebs buffer in which oxygen was continuously bubbled[7]. Each cornea was placed between the donor and the acceptor chamber of diffusion cells and oxygen was bubbled into Krebs buffer in both chambers. To quantify the transport of the formulation through cornea, buffer of donor chamber was replaced by 5 mL of formulation (AmB content 250 ug/ml). In another setup buffer of donor chamber was replaced by plain drug solution with similar drug content to compare the result. The goat corneas were removed after 1 hr., 3hrs., 6 hrs. and 12 hrs. respectively from the perfusion cells from both the setup. To quantify the corneal penetration of the drug buffer from the acceptor chamber was withdrawn at half an hour interval upto 12 hrs.

To study the corneal retention of AmB each tissue was rinsed with normal saline, blotted dry and transferred to pre weighed counting vials. The vials were reweighed and the weight of the tissues was calculated. The tissues were digested.The medium was acidified by using 1 ml of 1M HCl followed by centrifugation at 15,000 rpm for 15 min. The drug was extracted with DMSO and quantified by HPLC method.

A previously described HPLC method was used with some modifications as a reliable method of quantifying amphotericin B[8,9]. A stock solution of AmB was prepared by adding 10 mL of methanol to reach a final amphotericin B concentration of 5 mg/mL. Calibrations samples were prepared by first diluting this stock solution in a mixture of methanol/dimethylsulfoxide (DMSO): 50/50 (v/v). A second dilution was performed in Na_2EDTA 0.02 M /acetonitrile: 45/55 (v/v) at pH 5.0 run isocratically. Na_2EDTA 0.02 M /acetonitrile: 45/55 (v/v) was used as mobile phase with 5 pH which was flowing at a rate of 1 mL/min.

Results and Discussion

In vitro characterization

For RS 100 polymer, Particle size was in the range of 112-350 nm, entrapment efficiency was 26-72%, drug release at 12 hrs was 42- 89%. For RL 100 polymer, particle size range was 114-390 nm, entrapment efficiency was 30-72%, and drug release at 12 hrs was 50-90%. The details result is present in table 1.

From the result (table 2) of entrapment efficiency we concluded that increase in polymer weight and aqueous phase volume increased entrapment. This result is supported by the earlier result[10,11].

Release rate was higher for RL 100 formulations as it has higher water permeability. Almost 60-70% of the drug was released from every formulation in 6 to 7 hrs. Similar release pattern was also reported by Pignatello *et al*., 2002[5]. The release rate was related to polymer weight. Increase of drug

release was observed as a function of polymer weight upto 40 ml. Such finding can be related to progressive saturation of the polymer ammonium group by drug molecule occurring at higher polymer weight, which increases the dissolutive nature of drug release. This result is in support with the earlier studies[6]. But when polymer weight increased to 300 mg drug release decreased. This phenomenon may be due to highly increased polymeric concentration increases polymeric viscosity which worked as a thick barrier and hindered the drug release.

Ex vivo study

The Nanoparticles had better retention and more persistent interaction with the ocular surface compared to plain drug solution table 3. This result indicate two possibilities: First: Eudragit nanoparticle releases the drug in a sustained release manner. Second: It is evident that intact corneal tissues are less permeable than inflammatory tissue. If the drug is crossing the intact tissue it will surely cross the inflammatory tissues with a greater concentration. The similar results were reported earlier by Goldblum *et al.*, 2004. [12]

The prolonged ocular retention of the Nanoparticle compared to solution is in good agreement with a previous work that showed prolonged corneal retention of colloidal particles[13]. The results also specified that the concentration of AmB in cornea remained fairly constant for up to 6 h. Plain drug solution had less retention (20 ng at 1 hr.) and drug level was decreasing with increasing time (4.8 ng at 12 hrs). While almost 50 % and 85% of drug was penetrating at 12 hrs of nanoparticle of RS 100 and RL 100 formulation respectively. Penetration of drug was more incase of RL 100 nanoparticle may be due to higher permeation and zeta potential of this nanoparticles.

Conclusion

The goal to incorporate the large molecule of AmB into smaller nanoparticles and deliver them to corneal tissues was found satisfied. The *in vitro* results revealed excellent properties of nanoparticles including their entrapment efficiency. *Ex vivo* study on goat eyes exposed satisfactory result of drug penetration and retention at corneal tissues. However *in vivo* studies are mandatory to confirm these results.

Acknowledgement

I express my gratitude to Uttarakhand technical university for giving me the opportunity to carry out this work as part of my PhD.

References

1. Nayak N. (Fungal infections of the eye--laboratory diagnosis and treatment). *Nepal Med Coll J*, 2008; 10(1):48-63.
2. Sowka JW, Gurwood AS, Kabat AG. Fungal Keratitis. In: Hand Book of Ocular Disease Management. Review of Optometry.2009.
3. Goldblum D, Rohrer K, Frueh BE, Theurillat R, Thormann W and Zimmerli S, (Ocular Distribution of Intravenously Administered Lipid Formulations of Amphotericin B in a Rabbit Model). Antimicrobial agents and chemotherapy, 2002; 46 (12): 3719–3723.
4. Calvo P, Vila-Jato JL, Alonso MJ, (Evaluation of cationic polymer-coated nanocapsules as ocular drug carriers). *International Journal of Pharmaceutics*, 1997; 153: 41–50.
5. Pignatello R, Bucolo C, Ferrara P, Maltese A , Puleo A, Puglisi G, (Eudragit RS100® nanosuspensions for the ophthalmic controlled delivery of ibuprofen). *Eur J Pharm Sci*, 2002; 16: 53-61.
6. Pignatello R, Bucolo C, Spedalieri G, Maltese A, Puglisi G, (Flurbiprofen-loaded acrylate polymer nanosuspensions for ophthalmic application). Biomaterials, 2002; 23: 3247-3255.

7. Hall R, Andrews PL, Hoyle CH, (Effects of testosterone on neuromuscular transmission in rat isolated urinary bladder). *EJP*, 2002; 449: 301-309.
8. Alak A, Moy S, Bekersky.(A high-performance liquid chromatographic assay for the determination of amphotericin B serum concentrations after the administration of Ambisome, a liposomal amphotericin B formulation). Ther. *Drug Monit*, 1996; 18: 604–609.
9. Eldem T, Arican-Cellat N. (High performance liquid chromatographic determination of amphotericin B in a liposomal pharmaceutical product and validation of the assay). *J Chromatogr Sci*, 2000; 38: 338– 344.
10. Budhian A, Siegel SJ, Winey KI. (Haloperidol loaded PLGA nanoparticles: Systematic study of particle size and drug content). *Int. J Pharm.*, 2007; 336: 367-375.
11. Subedi RK, Kang KW, Choi HK. (Preparation and characterization of solid lipid nanoparticles loaded with doxorubicin). *Eur J Pharm Sci.*, 2009; 37: 508–513.
12. Goldblum D, Rohrer K, Frueh BE, Theurillat R, Thormann W, Zimmerli S. (Corneal Concentrations following Systemic Administration of Amphotericin B and Its Lipid Preparations in a Rabbit Model). Ophthalmic Res, 2004; 36:172-176.
13. Calvo *et al*. (Novel hydrophilic chitosan–polyethylene oxide nanoparticles as protein carrier). J. Appl. Poly. Sci., 2002; 249:1–8.

 ISSN-2347-5447

Table 1. Different properties of nanoparticles

Formulation code	Eudragit RS100 (mg)	Eudragit RL100 (mg)	Aqueous phase volume (ml)	Particle size (nm)	PDI	Zeta potential (mV)	Remark
F1	100	-	80	112± 15	0.198± 0.7	19.3 ± 1	More aqueous phase volume, large particle size but Polydisperse particle
F2	100	-	20	230.4 ± 5	0.564 ± 0.2	20 ± 2	Small uniform particle
F3	200	-	80	184 ± 10	0.475 ± 0.4	22.5 ± 1.6	Size and zeta increased with increase polymer
F4	200	-	20	280.7 ± 1	0.362± 0.7	24.3 ± 1	Low particle size than F3 because of low aqueous phase volume
F5	300		80	248	>1	25 ± 1	Amorphous precipitate of nanoparticle
F6	300		20	350	1	27 ± 1	
F7	-	100	80	114.2 ± 4.5	0.255 ±0.20	28 ± 2	Zeta increased in RL due to presence of more ammonium gr.
F8	-	100	20	195± 14	0.850 ±0.25	+31.9 ± 0.30	Size decreased with decrease aqueous Volume
F9	-	200	80	126± 10	0.750 ± 1	+32.1 ± 0.22	Size increased with increased polymer and aqueous Volume
F10	-	200	20	214.0 ±10	0.966 ±0.28	+32 ± 0.8	Low particle size than F9 because of low aqueous Volume
F11	-	300	80	220	1	+33 ± 0.5	Amorphous precipitate of nanoparticle
F12	-	300	20	390	>1	+34 ± 0.7	

Table 2. Entrapment efficiency of different nanoparticle formulation

Formulation code	% entrapment	Remark
F1	40±1.8	Increased aqueous phase (80 ml) increased entrapment (40 %)
F2	26±2.5	Decreased aqueous phase (20 ml) decreased entrapment (26%)
F3	60±3	Increased in both polymer (200 mg) and aqueous phase (80 ml) highly increased entrapment (60%) as compare to F1, F2, F3
F4	40±2.2	Increased polymer (200 mg) increased entrapment as compare to F1 and F2 but decreased aqueous phase (20 ml) decreased entrapment
F5	72	Entrapment increased (72%) with increased polymer (300 mg) and aqueous phase (80 ml).
F6	50	Entrapment decreased as compare to F5 cause of decreased aqueous phase (20 ml)
F7	50±3	Increased aqueous phase (80 ml) increase entrapment (50%)
F8	30±2.5	Decreased aqueous phase (20 ml) decrease entrapment (30%)
F9	62±1	Increased both polymer (300 mg) and aqueous phase (80 ml) highly increase entrapment (62%) as compare to F7 (50%), F8 (30%)
F10	35±1.8	Entrapment decreased (35%) as compare to F9 as aqueous phase decreased (20 ml)
F11	72	Entrapment increase (72%) with increased in polymer weight (300 mg) and aqueous phase (80 ml).
F12	40	Entrapment decreased (40%) as compare to F11 as aqueous phase decreased (20 ml).

Table 3. Entrapment efficiency of different nanoparticle formulation

Time (h)	Amount of drug transported through cornea to buffer solution		
	Plain drug solution	RS 100 nanoparticle formulation	RL 100 nanoparticle formulation
0.5	28 ng	12.4 µg	40.5 µg
1	40 ng	50 µg	72 µg
2	65 ng	78 µg	93 µg
3 hrs	130 ng	90µg	116.2 µg
4 hrs	190 ng	142 µg	150.8 µg
6 hrs	230 ng	176µg	194 µg