Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Issue 2, 2012

Research Article

FORMULATION AND OPTIMIZATION OF OCULAR POLY-D, L-LACTIC ACID NANO DRUG DELIVERY SYSTEM OF AMPHOTERICIN-B USING BOX BEHNKEN DESIGN

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Received: 8 April 2011, Revised and Accepted: 18 Sep 2011

ABSTRACT

Eye is the most vital organ of body. Management of ophthalmic disease is limited by poor bioavailability and therapeutic response because high tear fluids turn over and dynamics cause rapid elimination of the drug from the eyes. There are various new drug delivery systems to improve ophthalmic bioavailability like nanosuspension, minidisk, liposomes, niosomes , ocusert, dendrimers etc. Development and optimization of nanoparticles colloidal suspensions containing amphotericin-b as potential ophthalmic drug delivery systems was carried out using interfacial deposition method (nanoprecipitation). Box-Behnken optimization design was used for the optimization procedure, with polylactide acid concentration (X_1), solvent to non-solvent ratio (X_2) and pluronic-F68 concentration (X_3) as the independent variables. The response variables were particles size and entrapment efficiency. These nanoparticles had average diameter of 108-290 nm and zeta potential of 20-31 mV. Entrapment efficiency was found to be in the range of 45-67 %. In vitro release of amphotericin-b at 37°C for eight hours showed fast release with a biphasic pattern characterized by a fast initial release, followed by a slower release. Antimicrobial assay results showed that minimum inhibitory concentration value of test formulations was observed to be 1 µg/mL at 48 hours which is slightly lower than antifungal activity of free amphotericin-b solution. In vivo experiments showed that, following topical instillation of nanosuspension to a rabbit's eye there was no irritation. From these results we can conclude that amphotericin-b nanosuspension can be proposed as a potential ophthalmic delivery system for the treatment of ocular fungal infections.

Keywords: Amphotericin-b; Poly-d,l-lactic acid; Box-Behnken; Nanosuspension; Ocular tolerability.

INTRODUCTION

Ocular drug delivery is one of the most fascinating and challenging tasks facing the pharmaceutical researchers. The complex anatomy of human eye renders this organ highly impervious to foreign substances. Over the past decades, a variety of ocular drug delivery systems, including controlled release of drug, drug targeting, and penetration enhancement of the drug, have been investigated. A significant challenge to the formulator is to overreach the protective barriers of the eye without causing permanent tissue damage. Development of newer, more sensitive diagnostic techniques and novel therapeutic agents continue to provide ocular delivery systems with high therapeutic efficacy. Traditional ophthalmic solution, suspension, and ointment dosage forms no longer constitute optimal therapy for various diseases [1].

Nanoparticles offers various advantages over traditional ocular dosage form like enhanced drug absorption due to longer residence time of nanoparticles on the corneal surface, reduction in the amount of dose, drug released is obtained for a prolonged period of time, reduction in systemic toxicity of drug, higher drug concentrations in the infected tissue, suitable for poorly water soluble drugs and smaller particles are better tolerated by patients than larger particles, therefore nanoparticles may represent auspicious drug carriers for ophthalmic applications [2,3].

Proper selection of the polymeric matrix is necessary in order to develop a successful nanoparticulate delivery system. Poly (d,l-lactide) (PLA), poly(epsilon-caprolactone) and poly(d,l-lactide-co-glycolide) have gained attention for the preparation of a wide variety of delivery systems (blends, films, microspheres, nanosuspension, nanospheres, pellets, etc.) due to their biodegradable and biocompatible properties[4,5].

Amphotericin-b (AmB) is a broad spectrum antifungal drug that is used for treatment of local fungal infection in eye caused by Candida, Fusarium, Curvularia and Aspergillus which can lead to serious ulceration of the cornea if not treated rapidly. The current treatment consists of 0.15% (w/v) AmB eye drops prepared from Fungizone®, containing deoxycholate, irritant for the cornea which may lead to reduction in patient compliance. Various researchers are working with its novel drug delivery systems [6]. Among them, liposome (AmBisome®)[7,8], is quite successful formulations, but the major disadvantage is instability.

Designing of dosage form with the minimum number of trials is very crucial for the pharmaceutical researchers. Statistical experimental designs are powerful, efficient and systematic tools in the design of pharmaceutical dosage forms, allowing a rational study of the influence of formulation and/or processing parameters on the selected responses with a shortening of the experiment time and an improvement in the research and development work [9].

Response surface methodology (RSM) is one of the most popular methods in the development and optimization of drug delivery systems. It involves the use of various types of experimental designs, generation of polynomial mathematical relationships and mapping of the response over the experimental domain to select the optimum formulation [10,11]. Box-Behnken statistical design [12], is one type of RSM designs that is an independent, rotatable or nearly rotatable, quadratic design having the treatment combinations at the midpoints of the edges of the process space and at the center [13,14,15]. Additionally, it requires fewer experimental runs and less time and thus provides a far more effective and cost effective technique than the conventional processes of formulation and optimization of dosage forms.

Accordingly, the objectives of the present study were to develop a mathematical model in order to deduce the adequate conditions to prepare colloidal nanosuspension of desired characteristics, which could improve therapeutic effect of ocular AmB. In vitro release, antimicrobial activity and in vivo ocular tolerability were also examined.

MATERIALS AND METHODS

Materials

Amphotericin-b and Polyethylenepolypropyleneglycol (Pluronic F68) were supplied by Fisher Scientific (Bartlesville, OK). Methanol, Acetone and Dimethyl sulfoxide (DMSO) were supplied by Sigma Aldrich (St. Louis, MO). Polylactide acid RES 203 (MW 16,000) was supplied by Lakeshore Biomaterials (Brimingham, AL). All other reagents and solvents used were of analytical grade.

Methods

Experimental design

Three-level three-factor Box-Behnken experimental design was created using Statgraphics® Centurion XV.I (Statpoint Technologies, Inc., Warrenton, VA). This design was used to evaluate the effects of selected independent variables on the responses, to characterize particle size and entrapment efficiency and to optimize the procedure. This design is suitable for exploration of quadratic response and for construction of second order polynomial models, thus helping to optimize the process by using a small number of experimental runs. For the three-level three-factor Box-Behnken experimental design, a total of 15 experimental runs are needed. The generated model contains quadratic terms explaining the non-linear nature of responses. This design also resolves the two factor interaction effects of individual terms and allows a mid level setting (0) for the combination of factors [16,17]. The design consists of replicated center points and a set of points lying at the mid points of each edge of the multidimensional cube that defines the region of interest. The model is of the following form:

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_1X_2 + A_5X_2X_3 + A_6X_1X_3 + A_7X_1^2 + A_8X_2^2 + E,$$

where Y is the measured response associated with each factor level combination; A_0 is an intercept; A_1-A_8 are the regression coefficients; X_1, X_2 and X_3 are the factors studied; and E is the error term [18].

Mathematical relationships were subsequently generated to study the relationship between the dependent and independent variables.

After generating the polynomial equations, the process was optimized for the response Y_1 (particle size) and Y_2 (entrapment efficiently). Optimization was performed to obtain the levels of X_1, X_2 and X_3, which minimize Y_1 and maximize Y_2. To verify these values, a new formulation was prepared according to the predicted levels of X_1, X_2 and X_3. The obtained responses (Y_1 and Y_2) were calculated.

Preparation of PLA nanosuspension

Solvent displacement process was used to prepare AmB nanoparticles [19]. The nanosuspensions were obtained in the presence of 0.1% AmB, at different polymer and surfactant concentration and different solvent to non-solvent ratios. Drug and polymer were co-dissolved in acetone and methanol mixture (3:1) at pH 3.5 and then slowly injected into water (non-solvent) containing different concentration of Pluronic F68, as hydrophilic surfactant under moderate magnetic stirring. Finally, the organic solvents were evaporated under reduced pressure at 58°C. The process variables involved in nanoparticles preparation is presented in Table I.

Table I: Independent and dependent variables levels for Box Behnken design

Independent variables	Level		
	Low	Middle	High
Polymer Concentration (X_1)	0.25%	0.5%	0.75%
Surfactant Concentration (X_2)	0.2%	0.4%	0.6%
Solvent to Non-solvent ratio (X_3)	1:2	1:3	1:4
Dependent variables	Level		
	Minimum	Maximum	Goal
Particle Size (Y_1)	108	290	Minimize
Entrapment efficiently (Y_2)	45	67	Maximize

Particle size and zeta potential analysis

The mean particle size and zeta potential of all formulations was determined by photon correlation spectroscopy (PCS) with a Zetasizer (Malvern Instruments Ltd, United Kingdom), equipped with the Malvern PCS software.

Morphology

The morphological examination of the nanoparticles was performed with a scanning electron microscope (SEM JEOL JSM-6400; JEOL, Tokyo, Japan).

Determination of drug entrapment efficiency

One milliliter of formulation was taken and dissolved in a minimum quantity of DMSO. This solution was centrifuged at 13000 rpm for 20 minutes. One milliliter of supernatant was taken and adjusted to 10 mL with methanol: water (1:1, vol/vol) system. From this stock solution, again 1 mL solution was withdrawn and adjusted to 10 mL. The solution was analyzed spectrophotometrically at 403 nm. Each experiment was repeated in triplicate. Percentage drug entrapment was determined by the following formula:

$$\text{Entrapment efficiently} = \frac{\text{Amount of AmB actually present in nanoparticle}}{\text{Amount of AmB actully used}} \times 100$$

In vitro release profile

AmB release from nanoparticles was evaluated using diffusion cells, whereby a dialysis membrane with a molecular weight cutoff (MWCO) of 12000 to 14000 Da (Spectrum Laboratories Inc., Rancho Dominguez, CA) separated the acceptor from the donor compartment, consisting of 20 mL of formulation. The acceptor compartment was filled with 20 mL simulated tear Fluid (STF) and stirred magnetically at 200 rpm. Temperature was maintained at 37 ±0.5°C.

At regular time intervals within eight hours, samples of 1 mL were withdrawn from the acceptor compartment and replaced by the same volume of fresh STF solution. All the experiments were carried out in the dark and were repeated in triplicate. The samples were analyzed spectrophotometrically at 403 nm.

Antimicrobial assay

Paper disk diffusion method [20], was used for detecting the antimicrobial activity of formula No 12 and optimum formula. It was determined by potato dextrose agar plates previously inoculated with 18 hours old broth culture in sterile distilled water of the test organisms (*Fusarium solani*). Sterilized paper disks (6 mm) were soaked in the formulation (after diluting with distilled water) and laid on the agar surface. Test plates were incubated for 72 hours at room temperature to obtain maximum growth.

Ocular tolerability test

The potential ocular irritancy and/or damaging effects of dosage forms were evaluated according to a modified Draize test [21,22], using a slit lamp. Four male albino rabbits (body weight 2 kg) were used in the experiment. They were obtained from the animal breeding house in the Pharmacology Department, Faculty of Pharmacy, Al-Azhar University (Cairo, Egypt). All the animal experiments were approved by the Animal Care and Use Committee of Al-Azhar University.

A 0.01 mL aliquot of the test substance was instilled directly into the cornea of the right eye every 30 minutes for six hours (12 treatments). Left eyes treated with distilled water served as a control. Condition of the ocular tissue was observed after 10 minutes, at six hours, and 24 hours after the end of the experiments. The congestion, swelling, and discharge of the conjunctiva were graded on a scale from 0 to 3, 0 to 4, and 0 to 3, respectively. Hyperemia and corneal opacity were graded on a scale from 0 to 4.

RESULT AND DISCUSSION

Preparation of nanosuspension

AmB nanoparticles were prepared in a single step by interfacial deposition method (nanoprecipitation). Nevertheless, several difficulties must still be overcome to successfully incorporate the drug into the nanoparticles. The main difficulty was the selection of an organic phase that was capable of solubilizing both AmB and the polymer. For nanoparticles preparation, acetone (a water miscible and low boiling point solvent) is the solvent of choice. However, the preparation of AmB loaded nanoparticles in acetone yielded an amorphous precipitate of non associated drug [23]. One possible solution was the use of co-solvents. Moreover, it has been described that AmB solubility in different solvents can be increased by acidification. Therefore, a co-solvent and acidic conditions were utilized to optimize the solubility of both AmB and the polymer. This nanoparticles preparation process, apparently simple, may involve complex interfacial hydrodynamic phenomena. The origin of the mechanism of nanoparticles formation could be then explained in terms of interfacial turbulence or spontaneous agitation of the interface between two unequilibrated liquid phases, involving flow, diffusion, and surface processes [24,25]. The process would then be governed by the well known Marangoni effect, wherein movement in an interface is caused by longitudinal variations of interfacial tension [26,27]. It was possible to prepare nanoparticles in the absence of any surfactant, but Pluronic F68, a highly aqueous soluble surfactant, was needed for physical stability of the nanoparticles suspension.

Development of polynomial equations

A Box-Behnken experimental design with three independent variables at three different levels was used to study the effect of independent variables on particle size and entrapment efficiency.

A Box-Behnken experimental design has the advantage of requiring fewer experiments (15 batches) than would a full factorial design (27 batches). Transformed values of all the batches along with their results are shown in Table II. Table III shows the observed and predicted values with residuals for all the batches. Dependent variable obtained at various levels of the three independent variables (X_1, X_2, and X_3) was subjected to multiple regressions to yield second order polynomial equations:

$$\text{Particle size}(Y_1) = 121.125 + 151X_1 - 11.875X_2 - 7.625X_3 + 252X_1^2 + 5X_1X_2 - 63X_1X_3 - 12.5X_2^2 + 5X_2X_3 + 2.5X_3^2 \quad (1)$$

$$\text{Entrapment efficiency}(Y_2) = 49.75 + 27.5X_1 - 3.75X_2 - 2.875X_3 + 10X_1^2 - 10X_1X_2 - 1X_1X_3 + 9.375X_2^2 + 0.0X_2X_3 + 0.125X_3^2 \quad (2)$$

Y_1 and Y_2 values measured for the different batches showed wide variation (i.e. values ranged from 108 to 290 nm for Y_1 and 45 to 67% for Y_2) which clearly indicate that the Y_1 and Y_2 value is strongly affected by the variables selected for the study. This is also reflected by the wide range of values for coefficients of the terms in equations.

The main effects of X_1, X_2, and X_3 represent the average result of changing one variable at a time from its low level to its high level. The interaction terms (X_1X_2, X_1X_3, X_2X_3 X_1^2, X_2^2, and X_3^2) show how Y_1 and Y_2 changes when two variables are simultaneously changed.

The negative sign for the coefficients in equation 1 and 2 indicates a negative effect on responses, while the positive sign indicate a positive effect.

Table II: Experimental matrix and results

Run	Independent variables			Responses		Zeta potential
	X_1	X_2	X_3	Y_1	Y_2	
1	0.5	0.6	1:4	155	53	26
2	0.5	0.4	1:3	165	55	28
3	0.75	0.4	1:2	290	67	24
4	0.25	0.4	1:2	140	51	23
5	0.75	0.2	1:3	230	64	21
6	0.25	0.4	1:4	108	45	26
7	0.75	0.6	1:3	228	63	29
8	0.5	0.4	1:3	165	55	25
9	0.25	0.6	1:3	130	49	24
10	0.5	0.2	1:2	181	58	28
11	0.5	0.6	1:2	178	57	22
12	0.75	0.4	1:4	195	60	27
13	0.5	0.2	1:4	154	54	20
14	0.25	0.2	1:3	133	48	31
15	0.5	0.4	1:3	165	55	26

X_1 PLA concentration, *X_2* Pluronic F68 concentration, *X_3* solvent to non-solvent ratio, *Y_1* particle size, *Y_2* entrapment efficiency.

Table III: Observed and predicted values of the responses in Box-Behnken design

Run	Particle size			Entrapment efficiently		
	Observed	Predicted	Residual	Observed	Predicted	Residual
F1	155.0	145.00	10.00	53.00	52.63	0.38
F2	165.0	165.00	0.00	55.00	55.00	0.00
F3	290.0	275.12	14.88	67.00	66.25	0.75
F4	140.0	135.63	4.38	51.00	50.50	0.50
F5	230.0	234.88	-4.88	64.00	64.38	-0.38
F6	108.0	122.88	-14.88	45.00	45.75	-0.75
F7	228.0	233.63	-5.63	63.00	62.88	0.13
F8	165.0	165.00	0.00	55.00	55.00	0.00
F9	130.0	125.13	4.88	49.00	48.63	0.38
F10	181.0	191.00	-10.00	58.00	58.38	-0.38
F11	178.0	187.25	-9.25	57.00	57.88	-0.88
F12	195.0	199.38	-4.38	60.00	60.50	-0.50
F13	154.0	144.75	9.25	54.00	53.13	0.88
F14	133.0	127.38	5.63	48.00	48.13	-0.13
F15	165.0	165.00	0.00	55.00	55.00	0.00

Table IV: Analysis of variance (ANOVA) of particle size

Source	Sum of Squares	Df	Mean Square	F-Ratio	*P*-Value
A:Polymer Concentration	23328.0	1	23328.0	121.15	0.0001
B:Surfactant Concentration	6.13	1	6.13	0.03	0.87
C:Solvent to Non solvent ratio	3916.13	1	3916.13	20.34	0.0063
AA	915.92	1	915.92	4.76	0.081
AB	0.25	1	0.25	0.00	0.97
AC	992.25	1	992.25	5.15	0.072
BB	0.92	1	0.92	0.00	0.95
BC	4.0	1	4.0	0.02	0.89
CC	23.08	1	23.08	0.12	0.74
Total error	962.75	5	192.55	---	---
Total (corr.)	30143.7	14	---	---	---

R-squared = 96.8061%, R-squared (adjusted for d.f.) = 91.0572 %, Standard Error of Est. = 13.8762, Mean absolute error = 6.53333

Table V: Analysis of variance (ANOVA) of entrapment efficiently

Source	Sum of Squares	Df	Mean Square	F-Ratio	*P*-Value
A:Polymer Concentration	465.13	1	465.13	620.17	0.0000
B:Surfactant Concentration	0.5	1	0.5	0.67	0.45
C:Solvent to Non solvent ratio	55.13	1	55.13	73.50	0.0004
AA	1.44	1	1.44	1.92	0.22
AB	1.0	1	1.0	1.33	0.31
AC	0.25	1	0.25	0.33	0.59
BB	0.52	1	0.52	0.69	0.44
BC	0.0	1	0.0	0.00	1.00
CC	0.06	1	0.06	0.08	0.79
Total error	3.75	5	0.75		
Total (corr.)	527.6	14			

R-squared = 99.2892 %, R-squared (adjusted for d.f.) = 98.0099 %, Standard Error of Est. = 0.866025, Mean absolute error = 0.4

Table (IV and V) showed the ANOVA studies for Y_1 and Y_2. The statistical significance of each effect was tested by comparing the mean square against an estimate of the experimental error. It was noted that X_1 and X_3 had *p*-value less than 0.05, indicating significance of these variables in prediction of Y_1 and Y_2.

The standardized effect of the independent variables and their interaction on the dependent variable was investigated by preparing a Pareto charts (Figure 1 and 2), which depicts the main effect of the independent variables and interactions with their relative significance on the Y_1 and Y_2. The length of each bar in the chart indicates the standardized effect of that factor on the responses. Factors remains inside the reference line, indicate that these terms contribute the least in prediction of Y_1 and Y_2.

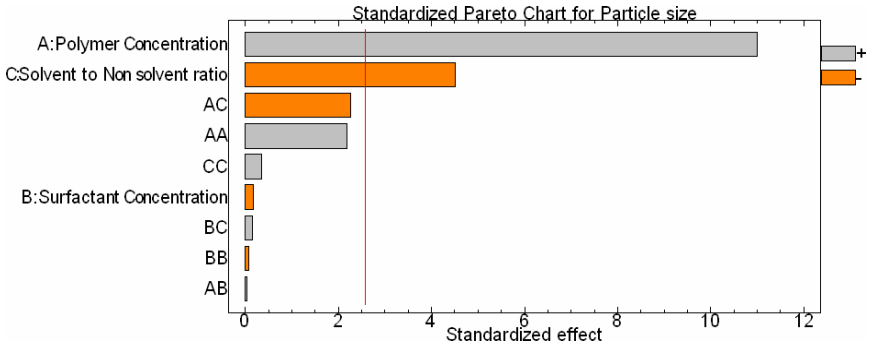

Fig. 1: Standardized pareto chart showing the effect of X_1, X_2 and X_3 on particle size

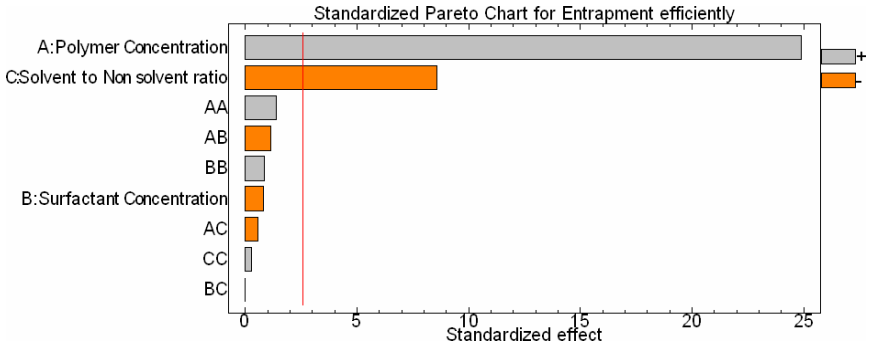

Fig. 2: Standardized pareto chart showing the effect of X_1, X_2 and X_3 on entrapment efficiency

The relationship between the dependent and independent variables was further elucidated by constructing contour plots and response surface plots.

Figure 3 is response surface plot and a contour plot for particle size that shows the effect of X_1 and X_3 at fixed level of X_2 (0.4%) on Y_1. Figures showed that at the high level of X_1 (0.75%), particle size decreased from 290 to 195 nm by increasing X_3 from 1:2 to 1:4. It was noted that at low level of X_3 (1:2), increasing of X_1 from 0.25 to 0.75%, results in increase particle size from 140 to 290 nm. The lines in the contour plot are nearly straight lines indicating no interaction between X_1 and X_3.

Figure 4 is response surface plot and a contour plot of entrapment efficiently that shows the effect of X_1 and X_3 at fixed level of X_2 (0.4%) on Y_2. The lines in the contour plot are nearly straight lines indicating no interaction between X_1 and X_3. From contour plot, if X_1 is 0.58% and X_3 is 1: 3.61 along with X_2 is 0.4%, entrapment efficiency will be 56.07%

Besides understanding the main and interaction effects on the responses, the experimental design approach is helpful in obtaining the optimized formula in which the levels of X_1, X_2 and X_3 were 0.7%, 0.2% and 1:4 respectively. In this instance, an optimized formula was theoretically obtained to yield particle size of 184.916 nm and entrapment efficiency 59.78%.

As a confirmation of this process, a new formulation was prepared at the optimum levels of the independent variables and evaluated. The observed value of responses for Y_1 and Y_2 were 181.23 nm and 60.23%, respectively, which give a close agreement with the predicted values.

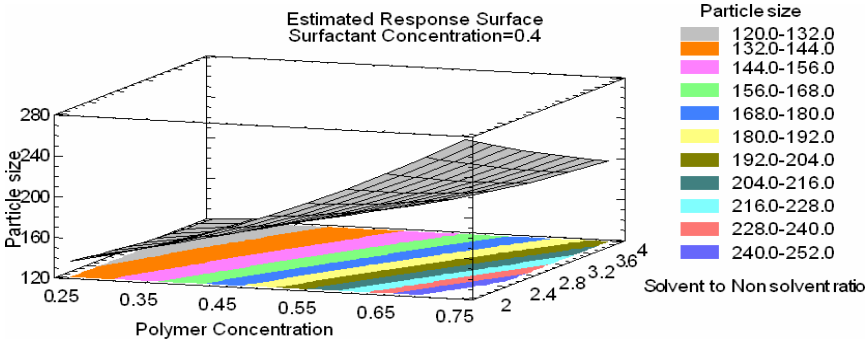

Fig. 3: Response surface and Contour plot showing the effect of X_1 and X_3 on particle size

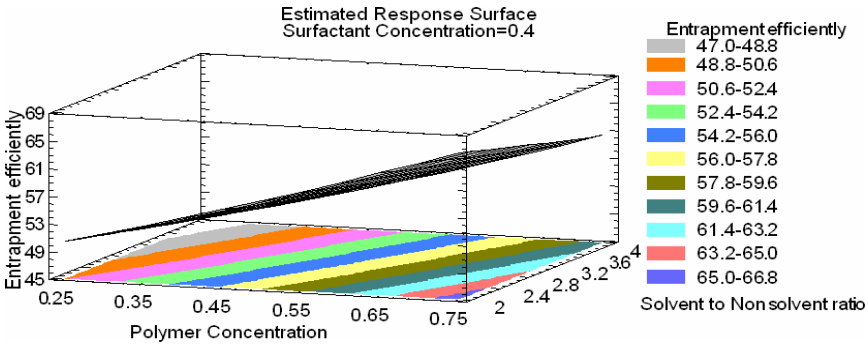

Fig. 4: Response surface and Contour plot showing the effect of X_1 and X_3 on entrapment efficiency

Particle size and zeta potential analysis

Particle size has a direct relevance to the stability and safety of such a formulation. Larger particles tend to aggregate to a greater extent compared to smaller particles, thereby resulting in sedimentation of nanoparticles. Particle size is crucial parameters for safe administration of such a formulation. Nanoparticles for ophthalmic application should not exceed 10 µm [28]. The mean particle size for drug loaded formulations varied from 108 to 290 nm (Table II). A graphical representation of the particle size of AmB nanoparticles obtained is given in Figure 5.

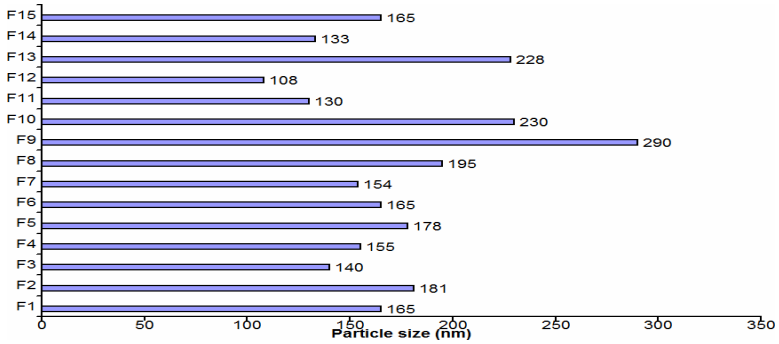

Fig. 5: Comparison of particle size of Box Behnken formulations

The results of this study show that the nanoparticles size is influenced by several formulative variables, with amounts of the polymer and solvent to non-solvent ratio being its main determinants.

Increase in polymer concentration leads to increase in particle size proportionately. This effect appears mainly to be due to the higher resultant organic phase viscosity, which leads to larger nanodroplets formation. This explanation is supported by the observation that larger sized nanoparticles were produced from a higher molecular weight polymer which also forms a more viscous organic solution [29].

Increase in aqueous phase volume decreases the particle size due to the increased diffusion of the water soluble solvent (acetone) in the aqueous phase [30].

Thus, larger particle size was obtained for formulations containing more polymer and less aqueous phase.

All AmB containing formulations showed a negative zeta potential value in the range of 20 to 31 mV (Table II). These data reflect the charges of native polymers.

Morphology

A SEM micrograph of AmB loaded nanoparticles showed that the particles have a uniform spherical shape with a smooth surface and are uniformly distributed (Figure 6).

Determination of drug entrapment efficiency

The drug entrapment efficiency varied from 45 to 67 % for the formulations prepared (Figure 7). The entrapment efficiency was affected by both polymer concentration and solvent to non-solvent ratio.

It has been shown that increase in polymer concentration will leads to increases in drug entrapment due to increase in organic phase viscosity, which increases the diffusional resistance to drug molecules from organic phase to aqueous phase, thereby entrapping more drugs in the polymer nanoparticles.

The solvent to non-solvent ratio is among the most critical parameters for the spontaneous formation of colloidal particles by the nanoprecipitation method [31]. An increase in the volume of the aqueous phase caused a decrease in the incorporation efficiency of AmB in the nanoparticles.

Previous data revealed that increasing non-solvent ratio leads to decrease in nanoparticles size. So it is reasonable to consider that the increase in the specific surface area caused by the formation of smaller nanodroplets may facilitate the diffusion of the drug to the external phase along with the solvent, leading to lower incorporation efficiencies [32,33]. It is also possible that the smaller the size of the nanoparticles, the lower the capacity of the polymer matrix to incorporate the drug.

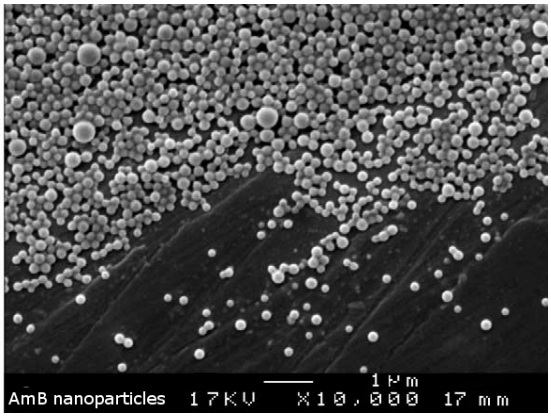

Fig. 6: Scanning electron micrograph of amphotericin-b loaded nanoparticles prepared by nanoprecipitation method.

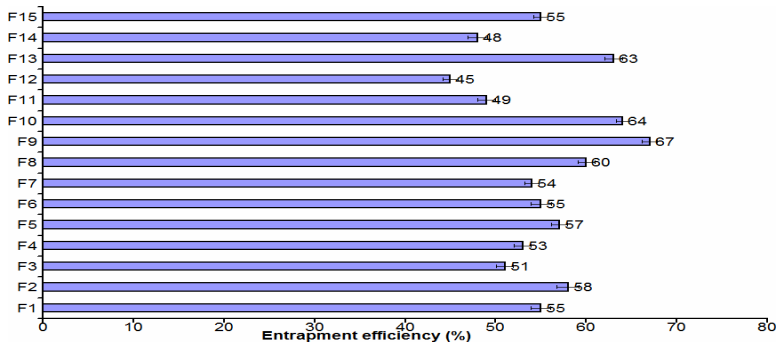

Fig. 7: Comparison of entrapment efficiency of Box Behnken formulations

In vitro release profile

The in vitro release experiments were carried out at 37°C because even though the corneal temperature is around 35°C (34.3°C at the center and 35°C at the periphery), the eye drop is instilled in the conjunctival fornix, where the temperature is 37°C. Drug release was monitored for only eight hours; a longer observation would have been useless for an ophthalmic application of these carriers because of the clearance of nanoparticles by lachrymal fluid.

Release of AmB from most formulation showed fast release independent of the processing condition (Figure 8).

The release behavior of AmB from the polymer matrix exhibited a biphasic pattern characterized by a fast initial release at the first, followed by a slower and continuous release of the drug. The burst release of AmB may be due to the dissolution and diffusion of the drug that was poorly entrapped in the polymer matrix located near the surface of nanoparticles, while the slower and continuous

release may be attributed to the diffusion of the drug localized in the PLA core of the nanoparticles.

Antimicrobial assay

The antifungal activity of AmB loaded nanoparticles was assessed in comparison to AmB solution and drug free formulation using a microbiological disk diffusion method. A linear curve was obtained for F12 and optimum formula in comparison to the control formulation (Figure 9).

This result predicts that test formulations possess slightly lower antifungal activity than free amphotericin-b solution on *F. solani*.

Drug free particles showed no antifungal activity at all, even though acid degradation products that were formed. Minimum inhibitory concentration (MIC) value of test formulations was observed to be 1 µg/mL at 48 hours. This is in compliance with the National Committee for Clinical Laboratory Standards (NCCLS) M38-A in vitro susceptibility data [34].

Ocular tolerability test

For a polymeric drug delivery to be proposed as an ophthalmic drug carrier, it is important to assay not only the biopharmaceutical properties but also the ocular tolerability. Therefore, in vivo ocular irritancy toward the AmB nanoparticles was determined following a modified Draize test protocol.

The in vivo results showed no sign of irritation or damaging effects to ocular tissues in rabbit eyes. The scores for conjunctival swelling and discharge were always zero. Iris hyperemia and corneal opacity scores were zero at all observations. The absence of in vivo irritant activity can promote the ophthalmic use of AmB nanoparticles colloidal carriers.

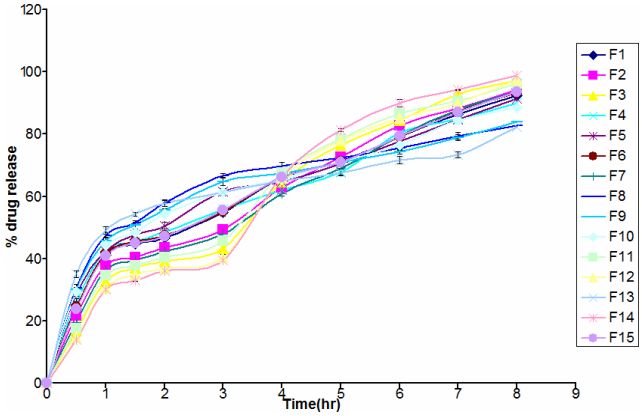

Fig. 8: Amphotericin-b release from various nanoparticles colloidal systems.

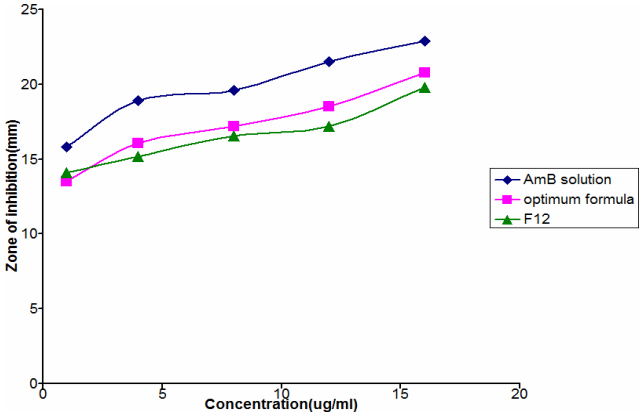

Fig. 9: Graphical comparison of zone of inhibition of two test formulations (optimum formula and F12) with Control (AmB solution) at 48 hrs.

CONCLUSION

These findings demonstrate that AmB can be entrapped in a polymeric colloidal drug delivery system using interfacial deposition method. The application of RSM gave a statistically systematic approach for the formulation of nanoparticles with desired particle size and entrapment efficiency. The results of antimicrobial activity as well as ocular tolerability, prompted us to use AmB-PLA-nanoparticles as a potential ophthalmic dosage delivery system for the treatment of ocular fungal infections, thus allowing a better compliance and an increased intraocular level of the antifungal agent.

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