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Research Article

"FORMULATION & PROCESS DEVELOPMENT OF AZITHROMYCIN OPHTHALMIC NANOSUSPENSION"

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ABSTRACT

Objective: The objective of this study was to prepare a novel stable azithromycin ophthalmic nanosuspension which has advantage over conventional ophthalmic suspension such as blurred vision, burning, stinging and irritation upon instillation. The viscosity was increased to provide additional advantage of long duration of action.

Method: Solvent diffusion method was used to prepare azithromycin ophthalmic nanosuspension.

Result: Average particle size of nanosuspension was 100 to 400 nm. Viscosity of prepared nanosuspension was 48 cps which is sufficient to give better retention with cornea. The *in-vitro* drug release study showed that the optimized nanosuspension released 92% of the drug within 8 hours.

Conclusion: It can be concluded from whole study that prepared nanosuspension was stable and non irritant with sustain release action.

Keywords: Nanosuspension, Ophthalmic delivery, High speed homogenization, Optimization, Azithromycin.

INTRODUCTION

Conjunctivitis, known as "pink eye," is an inflammation of the thin, transparent membrane covering the inner eyelid and the white part of the eye which is known as the conjunctiva. Conjunctivitis mainly classified in to five types i.e. viral pink eye, bacterial pink eye, allergic pink eye, chemical pink eye and Chlamydia pink eye [1]. Bacterial conjunctivitis usually is treated with antibiotic eye drops or ointment but is contagious as long as there is discharge from the eyes [2].

Azithromycin is a macrolide antibiotic and is active against gram-positive and gram-negative organisms [3]. Azithromycin is insoluble drug; hence preparation of nanosuspension can lead to colloidal dispersion having solution like properties with increased retention [4]. Addition of viscosity imparter is an additional advantage.

Ophthalmic nanosuspension can be defined as colloidal dispersions on nano-sized drug particles that are produced by suitable method and stabilized by a suitable stabilizer. These can prove to be a beneficial for drugs that exhibit poor solubility in lachrymal fluids [5, 6].

Mainly two types of techniques are available for preparation of nanosuspension, (1) Bottom up technique (2) Top down technique i.e. High pressure homogenization, lipid emulsion, media milling and dry co-grinding. High pressure homogenization is a reported technique for preparation of azithromycin nanosuspension [7]. The present research work was aimed to develop an optimized formulation & process for Azithromycin nanosuspension by solvent diffusion method.

MATERIALS & METHODS

Azithromycin was a gift-sample from Alembic pharmaceuticals, Baroda, India. Hydroxy propyl methyl cellulose E-5 (HPMC E-5) was purchased from Sulab laboratories, Baroda, India. Poloxamer 407 was purchased from Sigma life science, India. All other chemicals & reagents were of analytical grade.

Compatibility study

Compatibility of the azithromycin with HPMC E-5 and Poloxamer 407 used to formulate nanosuspension was established by Fourier Transformed Infrared spectral analysis. FT-IR spectral analysis of azithromycin and combination with HPMC E-5 and Poloxamer 407, kept at 60 ºC for 3 days, was carried out to investigate any change in chemical composition of the drug after combining it with the excipients.

Analytical method

In present study, Azithromycin was estimated by UV visible spectrophotometric method described in Chinese Pharmacopoeia [8], replacing water with artificial tear fluid (ATF). UV spectrum of this solution was recorded in the wavelength range 200-800 nm and the calibration curve for Azithromycin was prepared in pH 7.4 ATF as buffer.

Preparation of nanosuspension

Weighed quantity of azithromycin was dissolved in 5 ml organic solvent (Ethanol). This solution was added drop by drop using syringe fitted with 24-guage needle to 25 ml aqueous phase of HPMC E-5 and poloxamer 407 and homogenized using High speed homogenizer (Digital Ultra Turrax, Germany) [9] at 12,000-18,000 rpm for 10-20 min. This was followed by magnetic stirring for 2-3 hour to remove residual solvent.

Table 1: Formulation variables (3^2 factorial design).

Batch No.	Concentration of HPMC E-5 (X_1)		Concentration of poloxamer 407 (X_2)	
	Coded Values	Real Values (% w/v)	Coded Values	Real Values (% w/v)
FS1	-1	0.3	-1	0.1
FS2	-1	0.3	0	0.15
FS3	-1	0.3	+1	0.2
FS4	0	0.4	-1	01
FS5	0	0.4	0	0.15
FS6	0	0.4	+1	0.2
FS7	+1	0.5	-1	0.1
FS8	+1	0.5	0	0.15
FS9	+1	0.5	+1	0.2

Sterilization

Nanosuspension was prepared in sterile room. The formulation was filled in final container that was washed and rinsed with distilled water. Container Sealed with regular screw caps and sterilized at 121˚C for 20 min. [10].

Formulation optimization

The size of nanosuspension depends on the viscosity of medium & interfacial tension. Therefore, the amount of viscosity imparter (HPMC E-5) & surfactant (Poloxamer 407) were optimized using 3^2 factorial design. Nine batches were prepared using 3 different concentrations of HPMC E-5 & Poloxamer 407 (Table 1). Amount of all other ingredients were constant, i.e. 250 mg Azithromycin, 1.2% w/v Mannitol, 0.8% v/v HCl and 0.02% w/v benzalkonium chloride.

Process optimization

Process variables for high-speed homogenization are homogenization speed and homogenization time. Three levels for homogenization speed were selected within the range of 12000 to 18000 rpm and for homogenization time 10 to 20 min. based on trial experiments done in our lab.

Table 2: Process variables (3^2 factorial design).

Batch No.	Homogenization Speed X_1		Homogenization time X_2	
	Coded Values	Real Values (rpm)	Coded Values	Real Values (min.)
FP1	-1	12000	-1	10
FP2	-1	12000	0	15
F3P	-1	12000	+1	20
FP4	0	15000	-1	10
FP5	0	15000	0	15
FP6	0	15000	+1	20
FP7	+1	18000	-1	10
FP8	+1	18000	0	15
FP9	+1	18000	+1	20

Evaluation

pH

pH is an important property of ophthalmic formulation and should be maintain 7.4 to avoid irritation on application. Also stability of azithromycin is highly pH dependent [11]. pH of nanosuspension was measured by calibrated digital pH meter (Elico, LI 610).

Particle size and Polydispersity Index

Particle size of different formulations was measured with the help of Zetasizer (Malvern, UK) at 25°C. The average particle size diameter and polydispersity index of all formulations were measured.

Zeta potential measurement

Zeta potential of the formulations was also measured using Zetasizer (Malvern, UK) at 25°C.

Viscosity

Viscosity was measured by Brook field viscometer using spindle-61 at 25°C.

***In vitro* drug release:**

In vitro release of drug from the formulation was studied through Dialysis membrane -110 (HI-Media Laboratory Pvt. Ltd). Dialysis membrane was tied to one end of glass cylinder. Five milliliter of formulation was accurately placed in this assembly. The 50ml dissolution medium (Artificial Tear fluid) was stirred at low speed using magnetic stirrer [6, 12]. One milliliter dissolution samples were withdrawn at 1 hour interval for 8 hours and analyzed by UV-Visible spectrophotometer at 482 nm.

Evaluation of optimized batch

Kinetic Modeling

In order to understand the kinetic and mechanism of drug release, the result of in vitro drug release study of optimized nanosuspension batch were fitted into various kinetic equation like zero order (cumulative % release vs. time), first order (log % drug rang release vs. time) Higuchi's model (cumulative % drug release vs. square root of time), Korsmeyer-Peppas equation and Hixson-Crowell equation.

Sterility testing

Sterility testing was carried out by incubating formulations for 14 days at 30 to 35 °C in the fluid thioglycolate medium to find the growth of bacteria and at 20 to 25°C in soya bean-casein digest medium to find the growth of fungi in the formulation [13].

Surface morphology

Morphology of nano suspension was examined by Transmission electron microscope (TEM). The prepared nanosuspension was dropped onto carbon coated grid; extra solution was removed using a blotting paper. The grid was allowed to dry for 5 min. The TEM micrograph was taken by applying accelerating voltage of 80 kilo-volt [14].

Eye irritation study

Eye irritation study of nanosuspension was evaluated using isolated goat cornea. Whole eye balls of goat were obtained from local butcher. Eye balls were washed with cold saline to remove the proteins and then preserved in Krebs solution. In this study three eye balls were used. From three eye balls one was put in simple saline solution to get negative control, another eye ball was put in formulation for 8 hours, and last eye ball was put in NaOH solution as positive control. With the help of histopathology lab (Baroda clinical laboratory) obtained T.S of three eye ball. Prepared slides are examined under inverted microscope [14].

Stability studies

FP9 batch of Azithromycin nanosuspension was subjected to short term stability study for a period of 1 month as per ICH guidelines. In the present study, stability study was carried out at 40 ˚C ± 2 ˚C and 75% ± 5% relative humidity (RH). Nanosuspension was evaluated for particle size, pH, viscosity and % in vitro drug release [15].

RESULTS AND DISCUSSION

Drug-excipients compatibility studies

To study the compatibility of drug with excipients, IR spectra of pure drug and physical mixture of drug with all the excipients in 1:1 ratio was studied [16]. The peaks analyzed (Table 3) and IR spectra shown in Fig. 1 and 2 indicate that there was no physical and/or chemical interaction in between drug and studied excipients. The frequencies of functional groups of drug azithromycin remained intact in physical mixture containing different excipients. So it was concluded that there was no major interaction occurred.

Calibration curve of Azithromycin

The calibration curve of Azithromycin was prepared in artificial tear fluid at 482 nm. The linear plot obtained in artificial tear fluid had a correlation coefficient of 0.994, which followed Bear-Lambert's law in the concentration range of 20-100 µg/ml (Fig. 3).

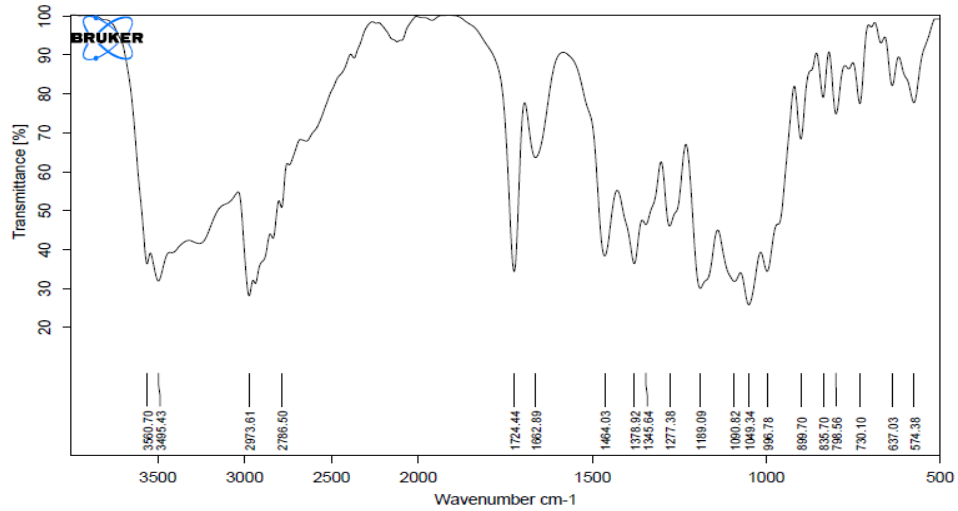

Fig. 1: FTIR Spectrum of Azithromycin

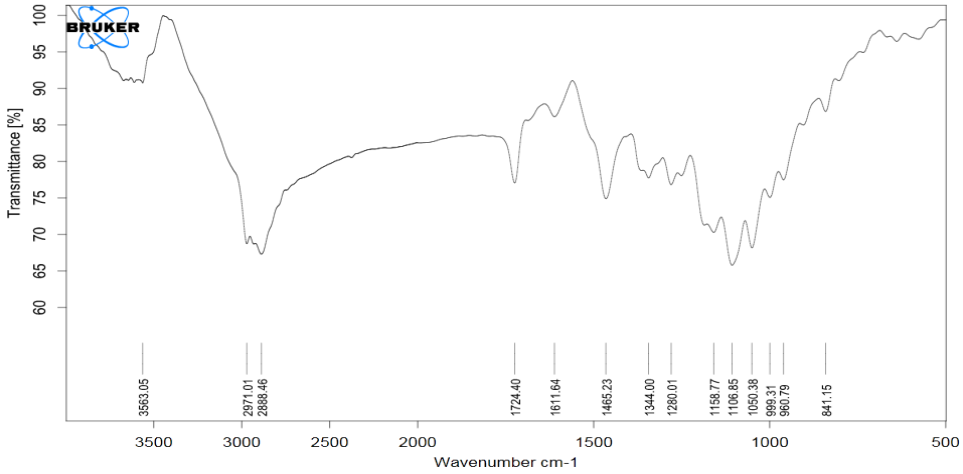

Fig. 2: FTIR Spectrum of Azithromycin + HPMC E-5 + Poloxamer 407.

Table 3: Comparison of FTIR Peak of Azithromycin and Physical Mixture after 3 day 60ºC

Observed peak in drug (cm^{-1})	Observed peak in mixture(cm^{-1})	Reported peak(cm^{-1})	Functional group	Interaction
3560	3563	3500-3700	-OH	No interaction
2973	2971	2800-3200	-CH3	No interaction
1724	1724	1705-1725	-C=O	No interaction
1189	1158	1000-1300	R-O-R	No interaction
1090	1106	1000-1350	C-N	No interaction

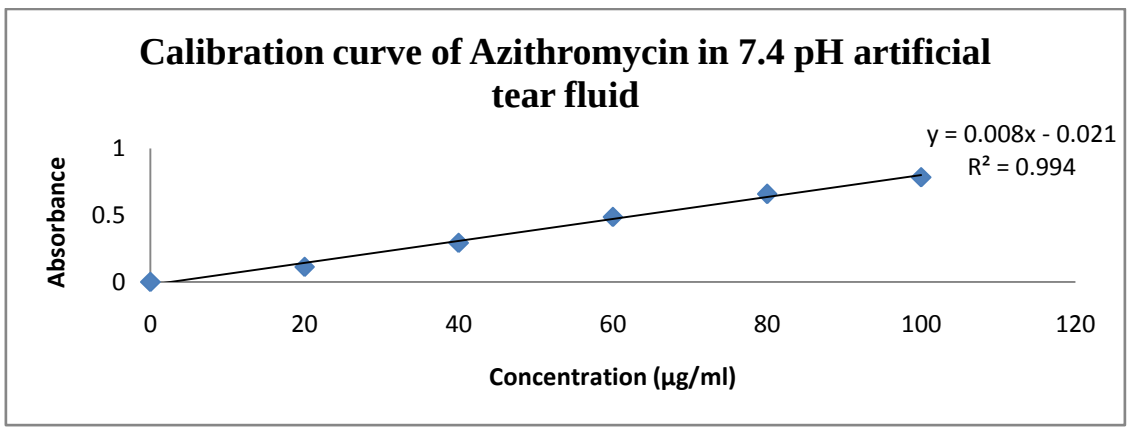

Fig. 3: Calibration curve of Azithromycin

Formulation optimization:

Evaluation of FS1 to FS9 was done by determining Particle size, size distribution, zeta potential, viscosity, and % *in vitro* drug release.

Particle size and polydispersity index

Particle size of the formulations was found in between 100–900 nm. With an increase in the concentration of stabilizer, decrease in particle size was observed. With an increase in the concentration of polymer, increase in particle size was observed. Formulations FS7 contain 0.3% HPMC E-5 and 0.2% poloxamer 407 showed lowest particle size (Table 4).

Polydispersity index (PDI) is the measure of size-distribution and varies from 0.0 to 1.0. The closer the PDI value to zero, the more homogenous is the nanosuspension. PDI of all formulations are shown in Table 4.

Zeta potential

Zeta potential of formulation FS1-FS9 was measured using Zetasizer (Malvern, UK). From the result of all the batches, formulation FS7 showed the zeta potential at 25 ˚C with highest zeta toward the negative side that was -21 mV. The high value of zeta potential indicates electrostatic repulsion between particles, zeta potential under ±30 mV shows good physical stability [4].

Viscosity

Viscosity measured by Brookfield viscometer with the help of spindle -61 at 25 ˚C. Viscosity of all formulation was within range of 45-58 cps (Table 4).

***In vitro* drug release study**

In vitro drug release study of FS1-FS9 was done to see the effect of formulation or process on the release pattern. Drug-release was found to be 71 to 85% in 8 hrs. FS7 batch showed maximum drug release 85% in 8 hours (Table 4).

From formulation optimization study, FS7 batch was found to be optimized batch having 0.5% w/v HPMC E-5 and 0.1% w/v poloxamer 407.

Table 4: Summary of evaluation of Batch FS1-FS9 for formulation optimization

Batch no	Particle size (nm)	Size distribution	Zeta potential (mV)	Viscosity (cps)	% *In vitro* drug release in 8 hours
FS1	539	1.0	-13	45	78
FS2	565	0.121	-12	48	72
FS3	821	1.0	-6	52	71
FS4	249	0.636	-17	48	80
FS5	320	0.520	-16	50	75
FS6	328	0.671	-14	52	72
FS7	113	0.259	-21	45	85
FS8	220	1.0	-20	55	83
FS9	250	0.513	-18	58	82

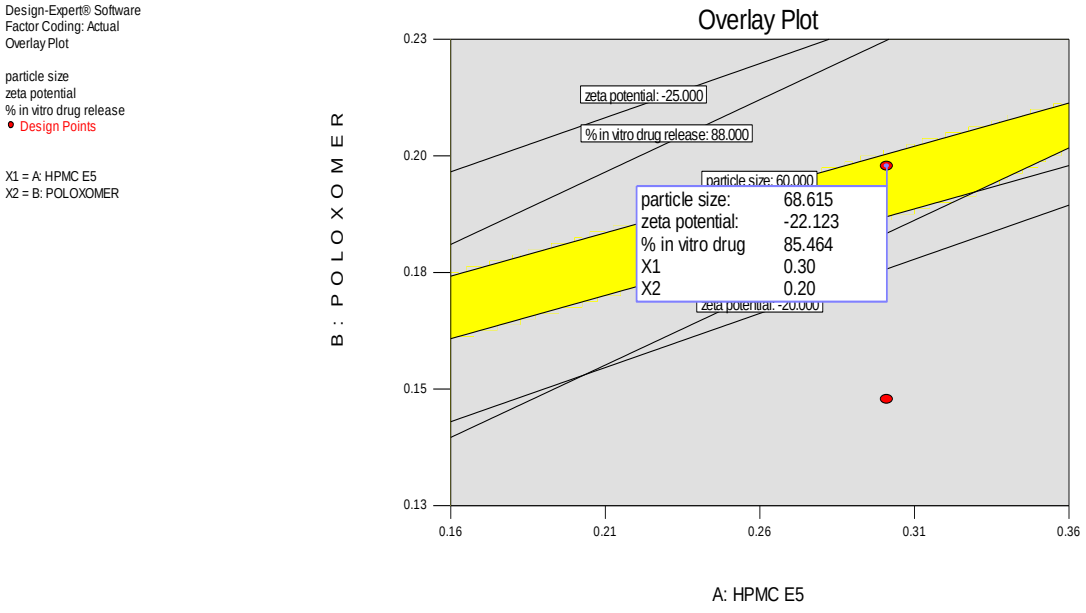

Fig. 4: Overlay plot for FS1-FS9

Overlay plot was prepared using Design Expert 8.0.7.1 software. From this overlay plot Particle size 68.6 nm, Zeta potential -22.1 mV, and *in vitro* drug release 85% in 8 hrs. It should come at the 0.3% w/v concentration of polymer HPMC E-5 and 0.2% w/v concentration Poloxamer 407 used as a stabilizer.

Process optimization:

Batches FP1-FP9 were prepared & evaluated for process optimization. In all batches concentration of HPMC E-5 (0.5% w/v) and Poloxamer 407 (0.1% w/v) was same as the optimized formulation batch FS7. From process optimization found that FP9 batch was optimized batch by evaluation of particle size, size distribution, zeta potential, viscosity, and % *In vitro* drug release study.

Particle size and polydispersity index

An increase the Homogenization speed and Homogenization time, decrease in particle size was observed. Formulation FP9 shows lowest particle size whereas formulation FP1 showed highest particle size.

Polydispersity index varies from 0.0 to 1.0. PDI of nanosuspension should be as low as possible for long term stability. PDI of all the formulations are shown in Table 5.

Zeta potential

Zeta potential of formulation FP1-FP9 was measured using Zetasizer (Malvern, UK). From the result of all the batches, formulation FP9 showed the zeta potential at 25 °C with highest zeta potential i.e. -33 mV.

Viscosity

Viscosity measured by Brookfield viscometer with the help of spindle-61 & found to be within range (48-56 cps) as shown in Table 5.

***In vitro* drug release study**

In vitro drug release study showed the % in vitro release profile of drug from FP9 was maximum, i.e. 92% in 8 hours (Table 5).

Release kinetic of optimized Batch FP9

The release kinetics data indicates that the release of drug from Batch FP9 follows first order drug release because the correlation coefficient values are higher in case of first order equation. The release rate is dependent on the time and indicates nanosuspension implies a surface action.

Table 5: Evaluation of formulation for process optimization

Batch no	Particle size (nm)	Size distribution	Zeta potential (mV)	Viscosity (cps)	% *In vitro* drug release in 8 hours
FP1	460	0.206	-12	52	72
FP2	374	0.59	-14	55	75
FP3	370	0.551	-12	51	76
FP4	341	0.007	-20	52	78
FP5	161	0.139	-15	50	82
FP6	157	0.759	-18	56	84
FP7	110	1.0	-19	49	87
FP8	92	0.063	-10	52	90
FP9	79	0.268	-33	48	92

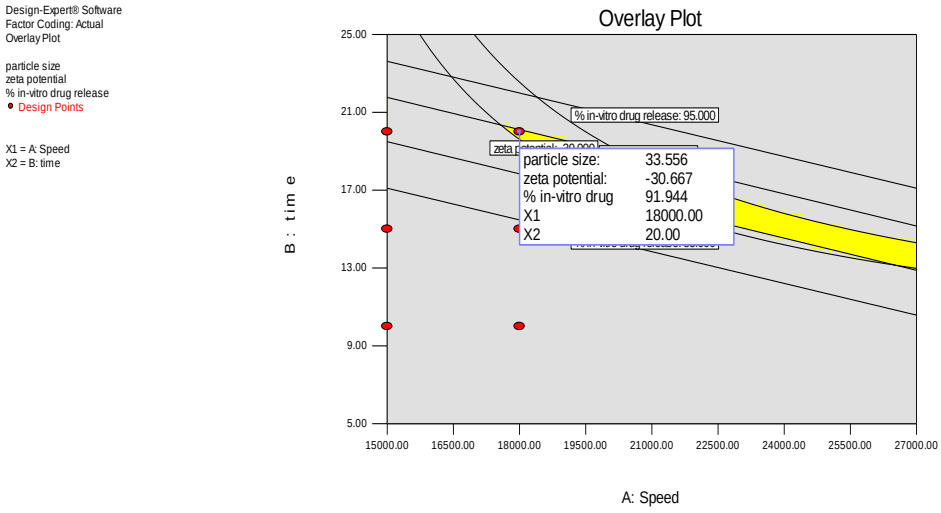

Fig. 5: Overlay plot for FP1-FP9

From this overlay plot, particle size 33.5 nm, Zeta potential -30.6 mV, and *in vitro* drug release 91% should be achieved at the 18000 rpm homogenization speed for 20 min.

Table 6: Optimized Formulation and Process parameter for azithromycin nanosuspension

Formulation	Azithromycin	250 mg
	HPMC-E5	75mg
	Poloxamer 407	50mg
Process	Homogenization Speed	18000 rpm
	Homogenization time	20 min.

Table 7: Summary of *in vitro* drug release of Optimized batch FP9

Time (hr)	Batch code FP9
1	7.080 ±0.115
2	13.669±0.056
3	22.604±0.115
4	35.339±0.072
5	44.716±0.060
6	60.263±0.119
7	74.898±0.058
8	92.798±0.065

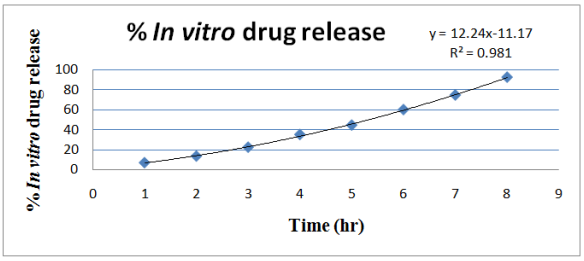

Fig. 6: *In vitro* drug release of FP9 batch in artificial tear fluid

Transmission Electron Microscopy

TEM (Fig. 7) of the optimized nanosuspension (FP9) showed that most of the nanoparticles exhibited spherical shape of drug. Size observed in TEM was 49.31 nm.

Sterility test

Formulation FP9 passed the test for sterility as there was no appearance of turbidity and hence no evidence of microbial growth when incubated for 14 days at 30-35 ° C in case of fluid thioglycolate medium and at 20-25 ˚C in the case of soya bean casein digest medium.

Eye irritation test

Irritation test was conducted on nanosuspension (FP9) to check possible irritation effect to the ocular tissue on *in-vivo* application. The microscopic images of ocular tissue showed blue colour in negative control (fig. 8A) and pink colour in positive control (fig. 8B) which showed hemorrhage. Test sample also showed blue colour (fig. 8C) so the investigated Azithromycin ophthalmic nanosuspension was classified as practically non-irritant.

Table 8: Release kinetic of optimized Batch FP9

Batch no	Zero order model	First order model	Higuchi model	Korsmeyer-peppas model	Hixson- Crowell model
	R^2	R^2	R^2	R^2	R^2
FP9	0.956	0.996	0.991	0.964	0.956

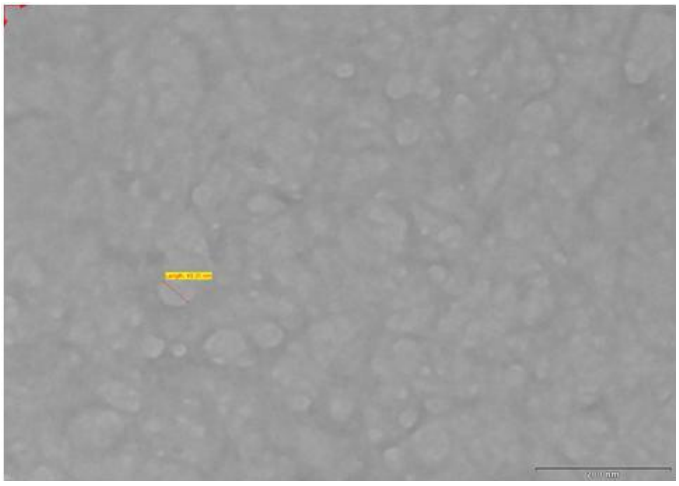

Fig. 7: Transmission electron microscopy of optimized batch FP9

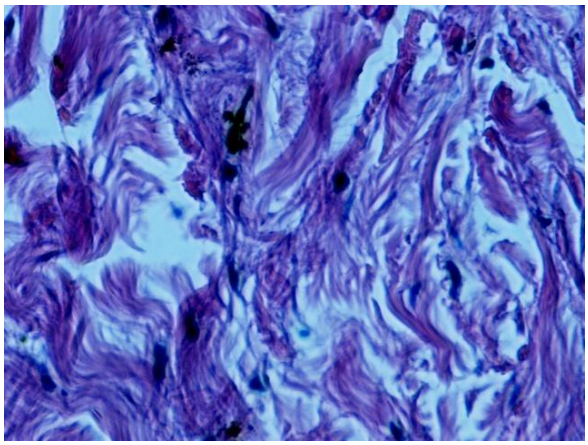

Fig. 8 A: Negative control

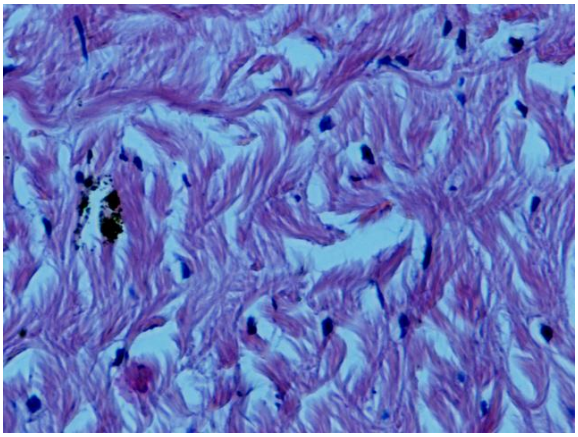

Fig. 8 B: Positive control

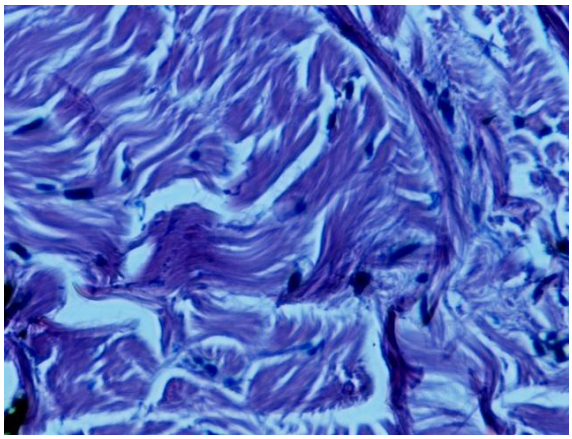

Fig. 8 C: Test sample

Stability study

Stability studies indicated that no significant changes were observed with respect to mean particle size, viscosity, pH, % *in-vitro* drug release, initially and after one month. It stated that, optimized batch was stable.

Table 9: Stability study of optimized batch no. FP9

Time (month)	Temperature/Humidity Condition	Mean particle size (nm)	Viscosity (cps)	pH	% *In vitro* drug release
Initial	Room temperature	79	50	7.4	92±0.80
1 month	40 ± 2°C / 75% ± 5% RH	86	52	7.32	91±0.73
1 month	Room temperature	82	51	7.38	91±0.61

DISCUSSION

Compatibility study was done by analyzing IR spectra of azithromycin and physical mixture of azithromycin with all excipients indicated that there was no physical and/chemical interaction in between azithromycin and studied excipients. Particle size was found to be 79 nm. Particle size of azithromycin nanosuspension increased with increased in concentration of polymer, which was different from the observation of Pandya *et al.* [17]. This may be due to increase in viscosity due to polymer concentration which makes decrease the mechanical impact needed for further size reduction. Zeta potential was found to be -33 mV which was within the range for stable formulation as stated by Bhavani *et al.* [15]. Zeta potential value of ± 20 mV is sufficient for stability of nanosuspension stabilized by poloxamer 407. Viscosity of the formulation was found to be 48 cps which was sufficient to give better retention time with cornea [10]. *In vitro* drug release study was found to be 92% in 8 hours followed first order kinetic which indicated sustained release pattern of formulation. Azithromycin ophthalmic nanosuspension passed the test for sterility as there was no appearance of turbidity and hence no evidence of microbial growth. This was similar to the observation of Mohanambal *et al.* [13]. Azithromycin ophthalmic nanosuspension passed eye irritation test as it was not showed hemorrhage after application in isolated got eye. So it was classified as practically non-irritant. Stability studies indicated that no significant changes were observed with respect to mean particle size, viscosity, pH and % *in-vitro* drug release, initially and after one month. It stated that, Azithromycin ophthalmic nanosuspension was stable.

CONCLUSION

The preparation of Azithromycin nanosuspension was attempted using high speed homogenization techniques to improve solubility of drug. The type of polymer and stabilizer used showed effect on the particle size of Azithromycin. TEM image showed spherical particles. No major drug polymer interaction was detected using FTIR. Ophthalmic nanosuspension may give better acceptance due to its small size, which may cause less irritation & blurring potential as compared to normal suspension. The prepared nanosuspension showed sustained action. The viscosity studies revealed that upon

simultaneous dilution with tear fluid viscosity drastically increased which may enhance ocular residence time drastically.

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