EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 3
Issue: 9
252-262
Year: 2016

DESIGN DEVELOPMENT AND EVALUATION OF NOVEL NANOEMULSION FORMULATION FOR OPHTHALMIC POTENTIAL OF INDOMETHACIN

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Article Received on 21/06/2016 **Article Revised on 12/07/2016** **Article Accepted on 04/08/2016**

ABSTRACT
The aim of this article was to formulate the non-steroidal anti-inflammatory drug Indomethacin as a ocular nanoemulsion of this high therapeutic efficacy and prolong effect. Nanoemulsion are potent drug delivery vehicles for ophthalmic use due to their numerous advantages sustained effect and high ability of drug penetration into the deeper layers of the ocular structure and the aqueous humor This system consisting of different oil, surfactants and co-surfactants were prepared by o/w type of nanoemulsion by high pressure homogenization. Castor oil nanoemulsion prepared with mixture of tween 80 and cremophor EL as surfactants together showed the fastest drug release among other castor oil nanoemulsion formulae. Indomethacin nanoemulsion were prepared and evaluated for the their physicochemical and drug release properties. These nanoemulsion showed acceptable physicochemical properties and exhibited slow drug release. Rabbit eye irritancy test that is Draize test and histological examination were carried out for those preparations exhibiting superior properties and revealed that they were non-irritant. Biological evaluation of indomethacin nanoemulsion on Albino wistar rabbits indicated that these products and the therapeutic efficacy. Prolong effect relative to either drug solution or the marketed formulation. Formulation of indomethacin in a nanoemulsion form offers, thus a more intensive treatment of inflammation of eye application per day, and a better patient compliance compared to conventional eye drop.

KEY WORDS: Indomethacin, inflammation, nanoemulsion, pharmacodynamics, physicochemical characterization.

INTRODUCTION
Ophthalmic drug delivery is one of the most interesting and challenging endeavors facing the pharmaceutical scientist[1]. It is a common knowledge that the application of eye drops as conventional ophthalmic delivery systems result in poor bioavailability and therapeutic response because of lacrimal secretion and nasolacrimal drainage in the eye [2,3]. Most of the drug is drained away from the precorneal area in few minutes. As a result, frequent instillation of concentrated solutions is needed to achieve the desired therapeutic effects [4]. But, by the tear drainage, the main part of the administered drug is transported via the nasolacrimal duct to the gastric intestinal tract where it may be absorbed, sometimes causing side effects [5]. In order to increase the effectiveness of the drug, a dosage form should be chosen which increases the contact time of the drug in the eye. This may then increase the bioavailability, reduce systemic absorption, and reduce the need for frequent administration leading to improved patient compliance. To overcome these problems, various ophthalmic vehicles such as suspensions, ointments, inserts, and aqueous gels have been investigated to extend the ocular residence time of medications for topical application to the eye [7]. These ocular drug delivery systems offer some improvement over conventional liquid dosage forms but, because of blurred vision (e.g., ointments) or lack of patient compliance (e.g., inserts), they have not been universally accepted. As a result, good ocular bioavailability following topical delivery of a drug to the eye remains a challenge yet to be resolved satisfactorily [8].

Inflammatory ocular disorders are usually treated with antibiotics, corticosteroids, non-steroidal anti-inflammatory and antiviral agents, etc. Many of these therapeutic agents face technological difficulties because they are poorly soluble in water and cannot penetrate easily biological membranes. This is particularly the case with drugs that are virtually insoluble in water and for that reason highly ineffective when used as liquid formulations (ocular liquid formulations).[7,9]

Ocular eye inflammation, one of the leading causes of irreversible ablepsia in the world, is estimated to be suffered by many people. It generally occurs in people

aged above 40, but it may also influence the visual acuity of much younger people, even children.[6] Topical delivery of drugs into the lower cul-de-sac using eye drops is a conventional approach for treatment and diagnosis of ocular diseases[1]. Many efforts have been made to enhance the efficacy of eye drops[5,6].

Novel drug delivery concepts and approaches are in high demand for improving the effectiveness, safety, and convenience of eye drops. Recently, nanoemulsion systems, especially the effective ones, have showed their potential in increasing efficacy and possible release of drug molecules for eye diseases because of their capacity to improve bioavailability of the drug and it is newer concept for ophthalmic solutions.

Indomethacin ([1-(4-chlorobenzoyl)-5-methoxy- 2-methylindol-3-yl] acetic acid), (IMC), is one such drug – it is for all practical purposes insoluble in water and unstable in acidic and alkaline solutions.[10]

For several years, nanoemulsions (NEs) have been investigated as new drug delivery systems, and their potential uses in ophthalmology have been studied by several research teams[11]. Formulations based on NEs have several interesting characteristics such as enhanced drug solubilisation, good thermodynamic stability and ease of preparation[12,13]. However, the most critical problem regarding the NE based drug carriers is the toxicity of the components. Recent efforts have been focused on how to decrease or eliminate the toxicity or irritation of the NE formulations[14]. The objective of the present study was to formulate Indomethacin in the form of NE for topical ocular administration of the drug using ocular safe components. The NE form is thought to offer a sustained release effect and high penetration of the drug through the cornea. This would therefore provide the advantages of conventional eye drops (ease of application and high patient compliance) and eliminate their disadvantages (low bioavailability and frequent administration).

MATERIALS AND METHODS

Materials

Indomethacin was received from Yaaro chemicals Pvt. Ltd. (Mumbai), India. Polysorbate 80, sodium chloride, sodium hydroxide, hydrochloric acid were obtained from Research Lab Fine Chem. (Mumbai). Cremophore EL and Castor oil was supplied by Pure chem. Laboratories, (Pune). All other chemicals were of analytical grade.

METHODS

Methods Determination of IMC saturated solubility in different NE components

To find out appropriate surfactants, cosurfactants and oils that have good solubilizing capacity of Indomethacin, the solubility of Indomethinac in various components was measured. Surfactants employed were tween 80, tween 20 and cremophor EL. Cosurfactants used were PG and transcutol P. Oils employed were IPM, oleic acid, peanut oil and castor oil.

Optimization by 3^2 Factorial Design[12,13,14]

Factorial designs are the designs of choice for simultaneous determination of the effects of several factors and their interactions. Factorial designs are used in experiments where the effects of different factors, or conditions, on experimental results are to be elucidated. This optimization method represents the mathematical technique which is an extension of classic method. This technique is applied to evaluate two or more factors that will affect the formulation development. There are some combinations of levels and factors, Factors are of two types first are dependent variables and second are independent variables and the number of experiments depends on the number of independent variables selected. Intervention studies with two or more categorical explanatory variables leading to a numerical outcome variable are called as "Factorial design." A factor is simply a categorical variable with 2 or more values referred to as levels .The study in which there are 2 factors with 3 levels is called as 3^2 factorial design. For the present work 3^2 factorial designs was selected and which is summarized in (Table No.1). In this design 2 factors are evaluated at 3 levels and experimental trials were carried out for all 9 possible batches and the combinations are reflected in (Table No.1). The two independent variables selected for the present study are Castor oil and Cremophore EL which is shown in (Table No.1).

Table No.1: Experimental Design as per 3^2Factorial Designs[64]

Formulation Code	Coded Values			
	X_1	%	X_2	%
F1	-1	14	-1	0.1
F2	0	16	-1	0.1
F3	-1	18	-1	0.1
F4	0	14	0	0.15
F5		16	0	0.15
F6	+1	18	0	0.15
F7	-1	14	+1	0.2
F8	0	16	+1	0.2
F9	+1	18	+1	0.2

Table No.2: Variables in Optimization Study

Veriables	Factor
Independent	
X1	Castor Oil
X2	Cremophor EL
Dependent	
Y1	In-vitro Drug Release
Y2	Anti inflammatory Activity

Methods of preparation of Nanoemulsion

The Oil Phase in that Indomethacin is solubalised in to the castor oil and that which kept as (Mixture1). Aqueous phase(Mixture2) in that Cremophore EL, polysorbate 80, NaCl which is mixed into the water and homogenized at 8000rpm by maintaining the temperature 60-70^{0}C. When homogenization takesplace the oil phase is directly poured into aqueous phase and homogenized.

Table No.3: Composition of Formulation Batches as per 3^{2}Factorial Design

Sr. no.	Ingredients	Formulation batches								
		F1	F2	F3	F4	F5	F6	F7	F8	F9
1	Drug	1	1	1	1	1	1	1	1	1
2	Castor Oil	1.5	1.5	1.5	2	2	2	2.5	2.5	2.5
3	Polysorbate 80	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
4	Cremphor EL	1	3.5	6	1	3.5	6	1	3.5	6
5	BKC	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
6	NaCl	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
7	HCL	1	1	1	1	1	1	1	1	1
8	NaoH	1	1	1	1	1	1	1	1	1
9	Dist.water	100	100	100	100	100	100	100	100	100

Selection of batch for optimization

From primary evaluation the F9 batch was selected because we got higher zeta potential value and lower particle size as well as lower poly-dispersity index.

Variation in the homogenization speed, time, temperature and its effect on particle size.

Procedure

Procedure for the preparation of nanoemulsion was same as performed above but the difference made for this batch was the speed and time for homogenization. The speed followed by time to be considered was 15,000 rpm, 20,000 rpm, and 25,000 rpm for 2hrs respectively.

Table no.4: Final batch selected for optimization.

Sr. no.	Excipients	F9 [5000 RPM] [2hrs]
1	Drug	1
2	Castor Oil	2.5
3	Polysorbate80	0.8
4	Cremophor EL	6
5	BKC	0.01
6	Nacl	0.6
7	HCl	1
8	NaOH	1
9	Distilled water	100ml

Physicochemical characterization of Nanoemulsions

Evaluation of rheological properties

Viscosity of the prepared formulae was determined using Brookfield Viscometer.

Volume

The volume of the formulation was chacked and was found to as per the required amount.

pH measurements

pH of the NE formulations was measured by Digital pH meter (Systronics, Ahmedabad).This was previously calibrated by pH 4 and pH 7. The pH values were recorded immediately after preparations.

Specific gravity

Specific gravity bottle is used to check the density of the formulation prepared and in turn compared with the density of water. Weight of empty gravity bottle is taken

as (M1), weight of specific gravity bottle containing the preparation is considered as (M2), weight of gravity bottle containing water is taken as (M3).

Centrifugation: (phase separation)
Nanoemulsions were subjected to centrifugations (Remi Motor) at 10,000 rpm for a period of 10 min and examined for any change in phase separation. Emulsions are thermodynamically unstable systems which may separate when subjected to physical stresses like centrifugation.. Result are shown in table no. 44. This confirmed the stability of nanoemulsion.

Particle size measurement[15]
Morphology and structure of the nanoemulsion can be studied using transmission electron microscopy. Droplet size distribution of the nanoemulsion can be determined by photon correlation spectroscopy that analyzes fluctuations in light scattering due to Brownian motion of the particles, using a Zeta seizer 1000 HS (Malvern Instruments, UK). The formulation (0.1ml) is dispersed in 50 ml of water, mixed thoroughly and light scattering was carried out at an angle of 90 at 25^0C.

Poly-dispersity[15,14]
The average diameters and poly-dispersity index of samples were measured by Photon Correlation Spectroscopy. The measurements should be performed at 25^0C.

Dilutability test[15]
O/W Nanoemulsion are dilutable with water whereas W/O are not and undergo phase inversion into O/W Nanoemulsion.

Drug content determination
Drug concentration in nanoemulsion solution was measured by spectrophotometer. Indomethacin content in solution was measured and added with known quantity of in solvent (Methanol) and it gets miscible. Absorbance was measured after suitable dilution at 319 nm in UV/VIS spectrophotometer (Jasco V-630, Japan) and % drug content was calculated.

***In Vivo* Corneal permeation studies using Goat Cornea[15]**
An Goat Ceornea were used to study the permeation across the coeneal membrane. Whole eye balls of goats were procured from a slaughter house and transported to a laboratory in normal saline maintained at 4^0C. The washed cornea were kept in cold freshly prepared solution of semulated tear fluid (STF) of pH 7.4. The study was carried out by modified Franz diffusion cell. A glass cylinder with both ends open, 10 cm height, 3.7 cm outer diameter and 3.1 cm inner diameter was used as permeation cell. The permeation study was carried out for 8hrs and sample were analyzed for Indomethacin spectroscopically at 319nm. The results were expressed as ± S.D.

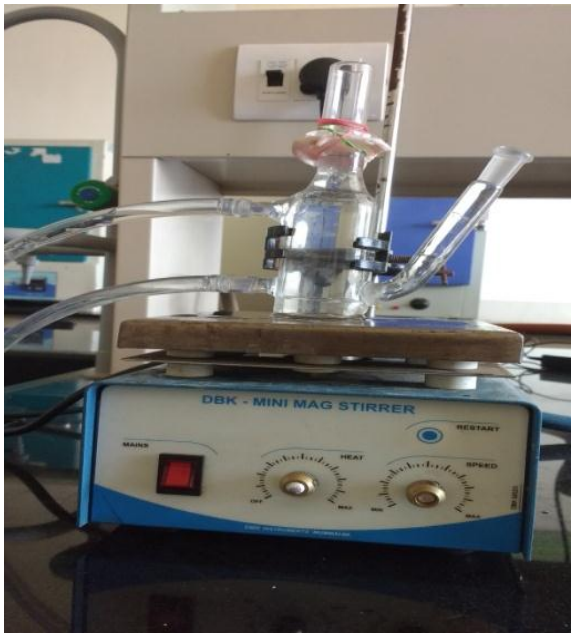


Figure 1: Modified Franz Diffusion Apparatus

Isotonicity Evaluation[14,15]
The formulations were mixed with few drops of diluted blood on a slide. The diluted blood was prepared by using Grower's Solution and slide was observed under microscope at 45x magnification. The shape of blood cells were compared with standard marketed ophthalmic formulation.

Test for Sterility [6,7]
Conditions for the test for sterility are given in Table no.23.

Table no.5: Test Conditions for sterility testing.

Test Conditions	
Method of sterility Test:	Direct Inoculation
Medium:	Sterile Fluid Thioglycolate, Artificial fluid Thioglycolate, Soyabean casein digest
Volume of individual test solution:	5ml
Positive control:	Sterilised media inoculated with *Staphylococcus Aureus*.
Negative control:	Sterile media
Incubation time:	14 days for detection of bacterial and fungal contamination.
Incubation Temerature:	20-25^{0}C bacteria
Method of detection:	Visual inspection of turbidity

Method: the sterility test was carried out as per IP (2014) method. The three medium were taken for this test that is fluid thioglcolate medium, artificial fluid thioglycolate medium and soyabean casein medium. The three set were prepared each set containing three tubes of each medium. The first set was a negative control for this sterile media is used, second set was a positive control for this sterilized media inoculated with staphylococcus aureus (MH1714) was used and third set was a test. The 1ml sterile optimized formulation was taken and this formulation was diluted with 100ml sterile water for injection, for this 5ml test solution was added in each medium. The formulation was incubated for not less than 14 days at 20-25^{0}C in the fluid thioglycolate medium and at 20-25^{0}C in soyabean casein digest medium to find out growth of bacteria in formulation.

7.7.13 Ocular irritancy test[14,15]

The test conditions for ocular irritancy test are shown in table no.6.

Table no.6: Test conditions for ocular irritancy test.

Test Conditions	
Method for test	Draize test
Strain of rabbit	New Zealand White Albino
Weight of rabbit	2-3 Kg
Volume of formulation instilled	50µL
Left eye of rabbit	Sterile water for injection
Right eye of rabbit	Optimized formulation(F9)

Rabbit eye irritation study shown in figure no.17.

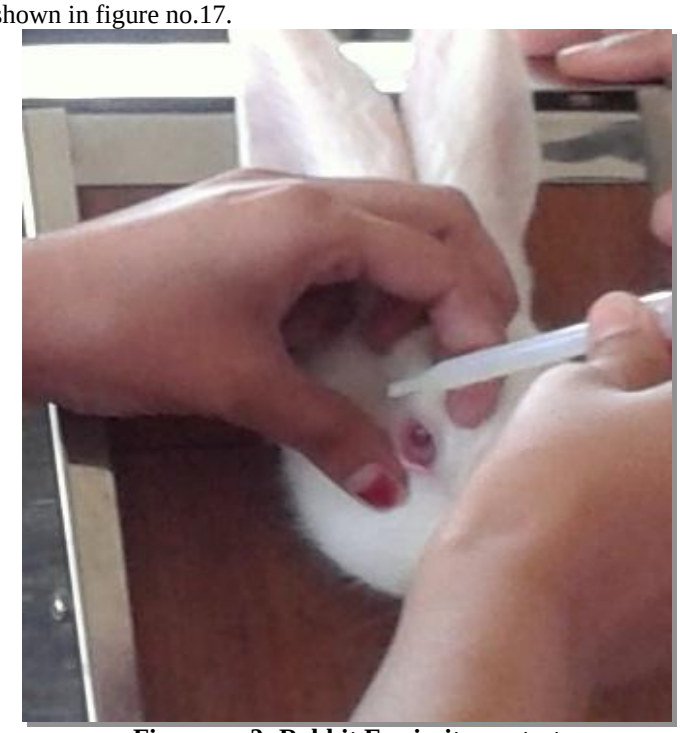


Figure no.2: Rabbit Eye irritancy test

The optimized formulation (F9) was used for eye irritancy study. The protocol was approved by Institutional Animal Ethics Committee with approval No.[IAEC/2015-2016/061516]

The rabbit was observed periodically for redness, swelling, watering of the eye. The score point was recorded as per the chart given in table no.26.

Table no.7: Score rating for eye irritancy study.

Sr. no.	Score	Rating
1.	0	None
2.	1	Slight
3.	2	Mild
4.	3	Moderate
5.	4	Severe

Thermodynamic stability studies[15]

Freeze-thaw cycle

Freeze thaw cycle was performed for four weeks. Formulation was kept at 5^0C for 1 week and later change over by keeping the same formulation at 40^0C in next week followed alternately with temperature.

Heating cooling cycle

This test was performed for 1 week and the temperature was changed every day in between 25^0C- 40^0C. Here 25^0C was considered as room temperature and 40^0C was set in oven. It was further evaluated for organoleptic properties and phase inversion.

Centrifugation

The nanoemulsion based solution was subjected to centrifugation studies where they were made to undergo centrifugation for 30 minutes at 5,000 rpm in a centrifuge machine. It was checked for phase separation or turbidity.

Stress testing

In this testing formulation was kept at 60^0C for one month.

RESULT AND DISCUSSION

1) pH Measurement: The ideal pH for maximum comfort when an ophthalmic preparation is instilled in the eye should be in the range of 7.2 ± 0.2. In some cases, the instillation of a solution with a pH different from tears causes irritation and painful sensation and the pH of all formulation from F1-F9 was found to be the range of 6.33-6.83. pH values of all formulation shown in Table no.8.

2) Viscosity: It has been assumed that ophthalmic instillation of a formulation should influence the normal behaviour of tears. Systems with low viscosity allow good tolerance with little blinking pain. In contrast, systems with enhanced viscosity, although less tolerant, induce an increase in ocular contact time by reducing the drainage rate and, as a consequence, improve bioavailability. The viscosity of the all formulation shown in Table no.8. The viscosity of all NE formulae decreased upon dilution with aqueous phase.

3) Phase separation: Nanoemulsions is a homogeneous single-phase systems they were subjected to centrifugation to confirm absence of No phase separation. None of the nanoemulsion showed signs of No phase separation of all formulation F1-F9 on centrifugation at 3000 rpm for 2 hours.

4) Specific gravity: Specific gravity of formulation were found 1.078.

5) Particle size measurement: The particle size of all formulation F1-F9 were found 394.06-44.06. The particle size were found good and poly-dispersity index of all formulation shown in table no.8. The particle size and PDI are shown in figure no.3 and zeta potential is shown in figure no.4.

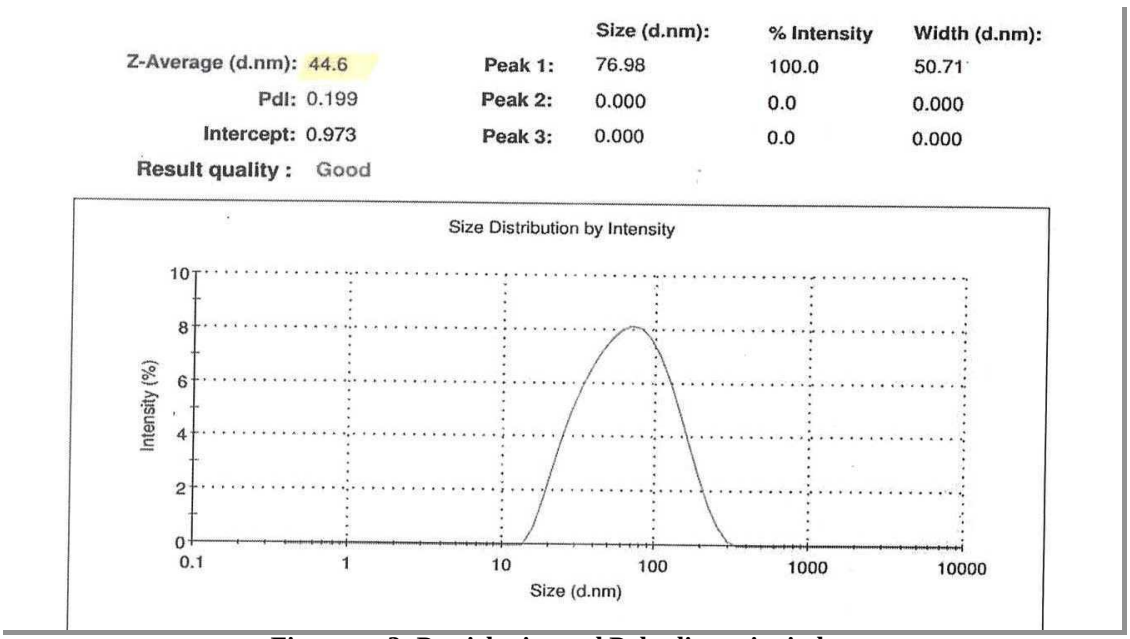


Figure no.3: Particle size and Poly-dispersity index

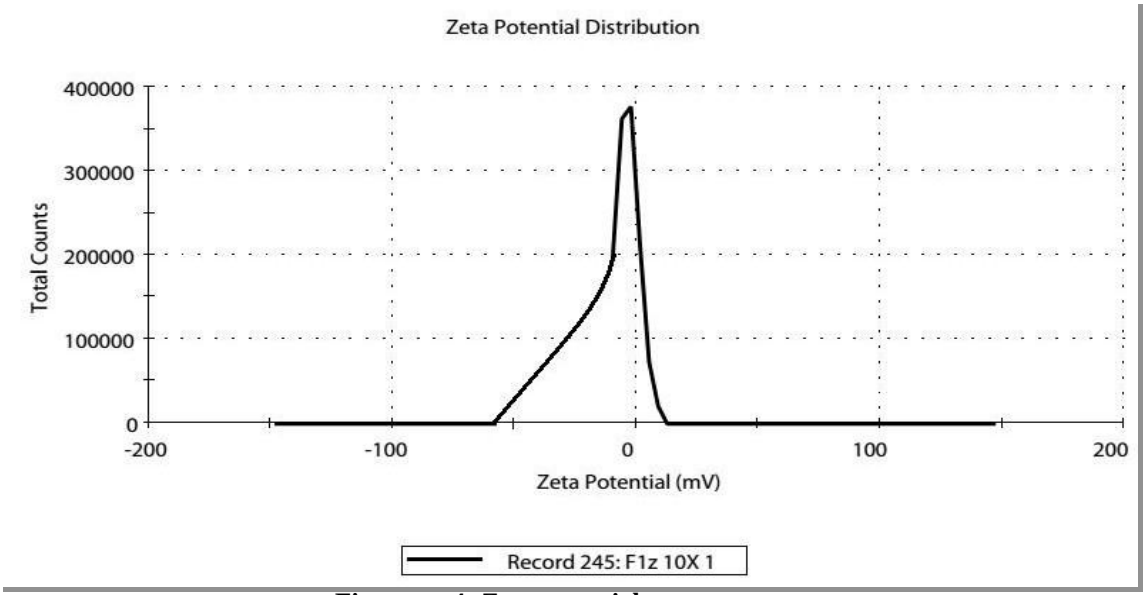


Figure no.4: Zeta potential measurement

6) Drug content: Drug content of all formulation F1-F9 shown in Table no.8.

7) *In Vivo* Corneal permeation studies using Goat Cornea

It was found that the increase in water content results in a decrease in permeation. This may be attributed to the decrease in concentration of surfactants and cosurfactant in NE which act as permeation enhancers by increasing the membrane permeability and consequently the drug uptake. % CDR of prepared formulation and marketed formulation are shown in figure no. 5.

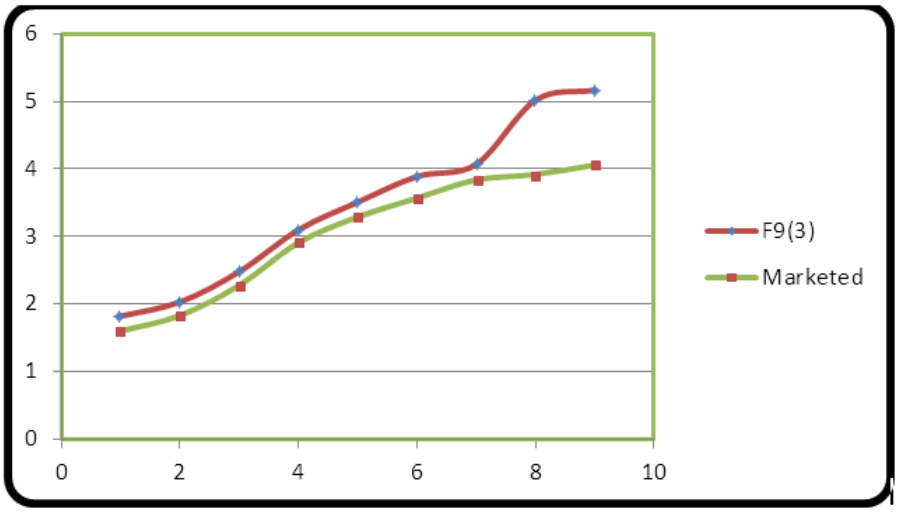


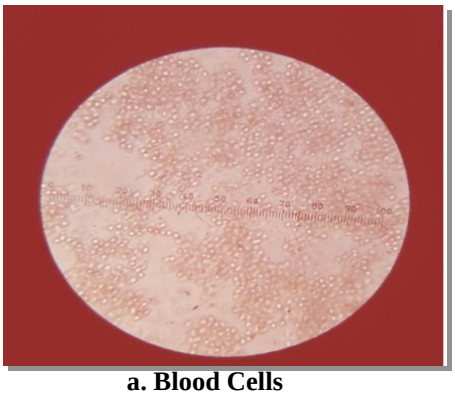
Figure no.5: %CDR of F9 (3) and Marketed formulation

Table No.8: Evaluation Parameters

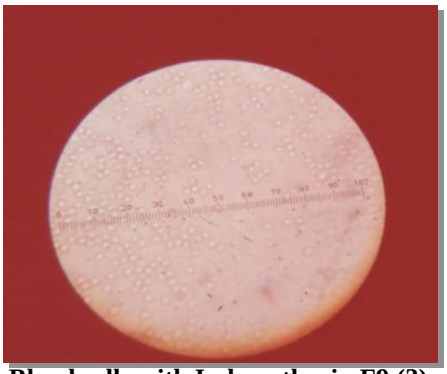
Sr. No.	pH	Viscosity	Phase separation	Particle size	PDI	Drug content	% CDR (8hrs)
1	6.33±0.1	28.156±0.2	No Phase separation	394.6	0.513	96.89 ± 0.002	1.82±0.0001
2	6.76±0.2	29.293±0.3	No Phase separation	264.3	0.465	97.45 ±0.0003	2.03±0.0006
3	6.45±0.1	29.177±0.3	No Phase separation	106.2	0.435	97.22±0.0005	2.48±0.0003
4	6.73±0.2	29.802±0.2	No Phase separation	103.8	0.404	97.53± 0.0003	3.10±0.0003
5	6.46±0.2	28.478±0.3	No Phase separation	100.2	0.375	97.73 ±0.0002	3.51±0.0001
6	6.36±0.3	29.38±0.3	No Phase separation	87.48	0.345	97.56 ±0.0011	3.89±0.001
7	6.76±0.2	29.56±0.2	No Phase separation	75.56	0.315	98.35 ±0.007	4.07±0.0001
8	6.76±0.2	29.77±0.2	No Phase separation	54.06	0.285	98.44± 0.002	5.02±0.0001
9	6.83±0.2	29.93±0.1	No Phase separation	44.06	0.199	98.65 ± 0.006	5.17±0.0001

8) Isotonicity Evaluation

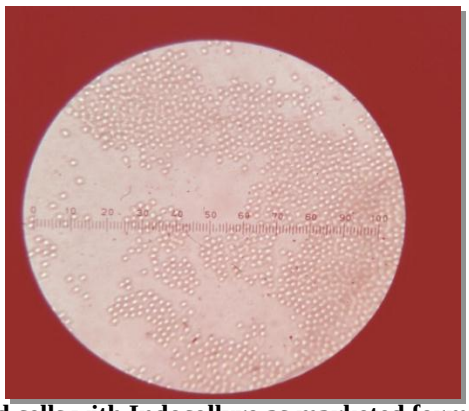
The shape of blood cells, blood cells with Indomethacin formulation F9(3) and blood cells with as a marketed formulation are shown in Figure no.6.



a. Blood Cells



b. Blood cells with Indomethacin F9 (3)



c. Blood cells with Indocollyre as marketed formulation

Figure no. 6: Shape of Blood cells

9) Test for Sterility: There was no appearance of turbidity and hence no evidence of bacterial growth when optimized formulation was incubated for 14 days at 30°C to 35°C in caseof fluid thioglycolate medium and at 20°C to 25ºC in case of soyabean-casein digest medium. The preparations examined, therefore, passed the sterility test.

10) Ocular Irritancy Test

The observations of ocular irritancy are shown in Table no 54.

Table no.9: Observations of Ocular Irritancy Study

Time	Redness		Swelling		Watering	
	Rabbit					
	1	1	1	1	1	1
	Left Eye	Right Eye	Left Eye	Right Eye	Left Eye	Right Eye
At the time of instillation (0hr)	0	0	0	0	1	1
30min	0	0	0	0	0	0
1hr	0	0	0	0	0	0
4hr	0	0	0	0	0	0
24hr	0	0	0	0	0	0
48hr	0	0	0	0	0	0
72hr	0	0	0	0	0	0
1 week	0	0	0	0	0	0

The results of the ocular studies indicate that the formulation F9 was non-irritant and no ocular damage or abnormal clinical signs were visible. The ocular irritancy study on rabbit eye is shown in Figure no.7.

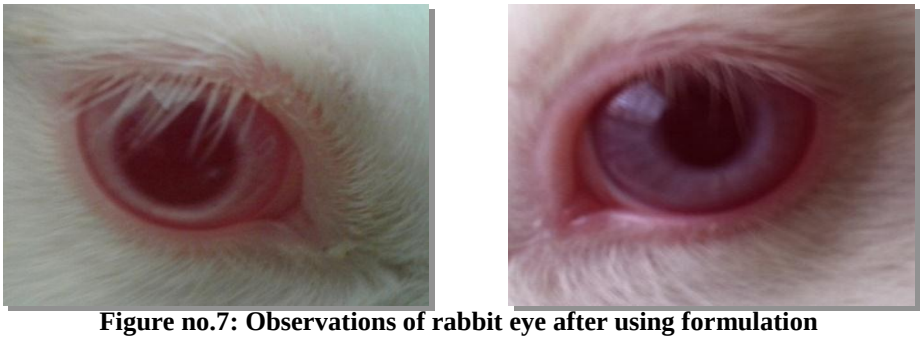


Figure no.7: Observations of rabbit eye after using formulation

11) Optimization

A 3^2 full factorial design was selected and the 2 factors were evaluated at 3 levels, respectively. The percentage of Castor oil (X_1) and Cremophore EL (X_2) were selected as independent variables and the dependent variable particle size determination and %CDR. The data obtained were treated using Design expert version 7.0.0 software and analyzed statistically using analysis of variance (ANOVA). The data were also subjected to 3-D response surface methodology to study the interaction of Castor oil (X_1) and Cremophore EL (X_2) on dependent variable. Table no.34 and table no.35 shows ANOVA for the dependent variable Particle size analysis and % CD respectively. The values of X_1 and X_2 were found to be significant at p <0, hence confirmed the significant effect of both the variables on the selected responses. From this data optimum concentration of castor oil and cremophore EL was found.

Final Equation in Terms of Actual Factors:

Y1 (Particle size Analysis) = + (498.84022)(112.07333)*(A)-(39.42800) *(B)

Final Equation in Terms of Actual Factors:

Y2 (% CDR) = + (29.04878) (19.48668)*(A) – (11.91933)*(B)

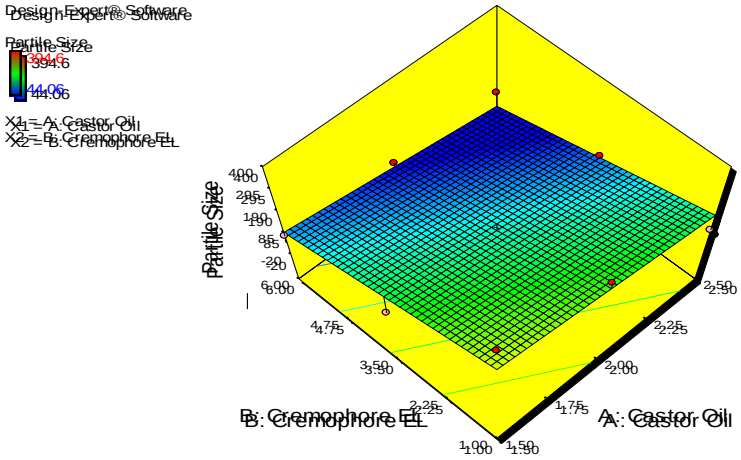


Figure no.8: Surface response plot showing effect of Castor oil and Cremophor EL on Particle size Analysis

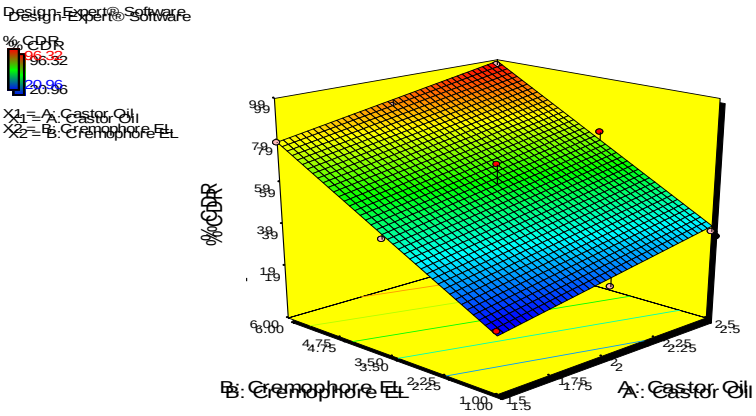


Figure no.9: Surface response plot showing effect of Castor oil and Cremophor EL on in vitro drug release.

12) Release Kinetics

The results showed that the factorial design batches followed korsmeyerpeppas model kinetics. The R^2 value of korsmeyer peppas model was found close to one as shown in Table no.54. The Drug Release Kinetics for best fitting optimized batch was calculated and it is shown in Table no.10.

Table no.10: Drug release kinetics for optimized batch

Sr. No.	Model Fitting	R^2 Value	N
1.	Korsmeyer- peppas	0.9912	0.6586

14) Stability study

Accelerated Stability study of optimized F5 formulation at temperature (40ºC±2ºC) *shown in Table no.10.

Table no.10: Stability study of Optimized formulation (F9-3)

Sr.No	Observation	Before Stability testing	After Stability testing		
			1 month	2 months	3 months
1.	Phase separation	Phase separation	No Phase separation	No Phase separation	No Phase separation
2.	Visual Appearance	Slightly White	Slightly white	Slightly White	Slightly White
3.	pH	6.83	6.83	6.82	6.81
4.	Drug content	98.52%	98.52%	98.52%	98.52%

Formulation at 40ºC±2ºC was found to be stable up to 3 months. There is no change in drug content, clarity and pH.

CONCLUSION

Indomethacin NEs were prepared successfully using Castor oil and cremophore EL at 39% water content. Indomethacin release rate from NEs were dependent on the type of surfactant and cosurfactant used in NE preparation. Formulation F9 showed highest drug release among other NEs. All prepared NEs showed acceptable physicochemical properties, thermodynamic stability and they were non irritant to rabbits' eyes. NEs improved Indomethacin permeation across goat corneas with 6 times improvement in permeation coefficient of Indomethacin when incorporated in formulation F9. Prolonged effect as well as enhanced drug bioavailability compared to Indomethacin eye drops. Thus, Indomethacin in NE F9 offers an intensive treatment of inflammation of eye with a decrease in number of instillation per day and a decrease or disappearance of systemic side effects of Indomethacin with an improvement in patient compliance.

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