CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

http://doi.org/10.5281/zenodo.1252391

Available online at: http://www.iajps.com Research Article

STABILITY AND OCULAR *IN-VIVO* PHARMACODYNAMIC STUDY OF ION ACTIVATED BRIMONIDINE TARTARATE *IN-SITU* GEL

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Abstract:

Objective: *The goal of this study was to evaluated anti-glaucoma activity of ion-activated in-situ gel of Brimonidine Tartarate (BT) and to predict the shelf of the developed formulation.*

Method: *sol-gel formulation was prepared by using gellan gum as an ion-activated gel-forming polymer, sterculia foetida gum and kappa carragennan as mucoadhesive agent and hydroxy propyl methyl cellulose (HPMC E50LV) as release retardant polymer. Phenyl ethyl alcohol as preservatives in borate buffer. Glaucoma was induced by marginal ear vein using 5% dextrose solution. Schiotz tonometer was use to measure the induced glaucoma. Long terms and accelerated stability studies were carried out. The formulation was characteristics for pH, clarity, sterility, in-vitro gelation studies and drug content by RP-HPLC method.*

Result*: The mean normotensive IOP was in the range of 17.5±0.08 to 19.1±0.40 mm Hg in both the eyes. Glaucoma was induced in the both eye with 5% dextrose solution and the mean IOP in both eyes was in the range of 26.2±0.21 to 29.9±0.25 mm Hg. Student paired T-test (F2) vs. Distilled water) was analysed and it was found that there is a highly significant difference between the means. This indicates the test drug is effective in the form of in-situ gels. The repeated measures one –way ANOVA with Tukey's multiple comparison test indicate the data is not significant, i.e p value is greater than p<0.05. In-situ gel are able to control the induced IOP and the IOP values of treated in-situ gel are near to normal IOP reading. Student unpaired and paired T test along with one way ANOVA data shows that there is a marginal significant difference between the means of F1 and F2. With p value of <0.0060 for student unpaired, <0.0050 for student paired T test. There is no significant difference between F2 and normal IOP but there is a marginal significant difference between F1 and normal IOP. Which highlight F2 is better in controlling induced/elevated IOP compared to F1. in-situ gels were translucent, Immediate gelation was formed with in sec, as it was dropped and remained stable. The formulations were free from micro-organisms at 30 °C ± 2 °C/65% RH ± 5% RH and at 40°C ± 2°C/75% RH ± 5% RH. The chromatograms were obtained with acceptable tailing factors (<2), not much variation was observed in the retention time. % drug content was found to be in the range of 99.46 % to 96% at 40°C ± 2°C/75% RH ± 5% RH by RP-HPLC. The shelf life (t90%) of F2 was found to be 2.5 years at 30 °C ± 2 °C/65% RH ± 5% RH .and at 40°C ± 2°C/75% RH ± 5% RH shelf life (t90%) was found to be 2 years.*

Conclusion: *5 % dextrose infusion through marginal ear vein is better in inducing IOP. Schiotz tonometer remains the preferred screening instrument. Brimonidine tartarate has a dual* mechanism of action *by reducing aqueous humor production and increasing uveoscleral outflow. So a* synergistic effect can be obtained with help of in-situ gels in order to control elevated intra-ocular pressure in glaucoma. *The above study highlights that formulation with sterculia foetida is better compared to kappa carragennan in controlling induced IOP.*

Key words: *Sterculia Foetida gum, marginal ear vein, Schiotz tonometer, Brimonidine Tartarate.*

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Please cite this article in press Vazir Ashfaq Ahmed and Divakar Goli., ***Stability and Ocular In-Vivo Pharmacodynamic Study of Ion Activated Brimonidine Tartarate In-Situ Gel,*** *Indo Am. J. P. Sci, 2018; 05(05).*

INTRODUCTION:

Glaucoma is the second leading cause of blindness. Worldwide, it is estimated that about 66.8 million people have visual impairment from glaucoma, with 6.7 million suffering from blindness, which will increase to 79.6 million by 2020.Glaucoma is characterized by slow progressive degeneration of the retinal ganglion cells (RGCs) and the optic nerve axons, leading to increasing deterioration of the visual field. If untreated, the condition can lead to irreversible blindness. Lowering the intraocular pressure will not restore lost vision, but controlling it, will prevent further vision being lost. Medication is the first management choice for most patients with glaucoma. Persistence and adherence to medication regimens is vital in the management of glaucoma. *In-situ* gels have major advantages like ease of administration, reduced frequency of administration, improved patient compliance and comfort [1-2]. The main advantages to using rabbits as experimental models in eye research are the large size of the rabbit eye and the several hundred years worth of accumulated data on the anatomy and physiology of the rabbit eye and its similarity to the human eye. Added to this, the fact that rabbits are easy to handle and breed and the most economical of the larger breed models, makes them ideal for ophthalmic research. Eye pressure is measured in millimeters of mercury (mm Hg). In human beings normal eye pressure ranges from 12-22 mm Hg, and eye pressure of greater than 22 mm Hg is considered higher than normal. When the IOP is higher than normal but the person does not show signs of glaucoma, this is referred to as ocular hypertension. The normal intraocular pressure of the rabbit is between 15 and 20 mmHg. Glaucoma is difficult to study in humans. The damage present at the time of diagnosis precludes the study of disease development from onset.[3] Additionally, obtaining retinas at equivalent pathologic states is rare, confounding comparisons and limiting conclusions. For these reasons, the development of animal models has become necessary for studying of the pathophysiology of glaucoma. Animal studies have articulated the mechanisms of the formation and evacuation of aqueous humor as well as the maintenance of intra-ocular pressure, thereby informing glaucoma etiology and therapeutic development.[4]

Stability is a critical quality attribute of pharmaceutical products; therefore stability testing plays a crucial role in the drug development process. The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light and to establish a retest period for drug substance or a shelf life for the drug product and recommend storage conditions. Therefore it encompasses all the phases of the drug development process. [5]

The aim of this work is to formulate Brimonidine Tartarate ocular *in situ* gelling system containing sterculia foetida gum as a mucoadhesive polymer, and to studies the effect of Brimonidine Tartarate on induced glaucoma on rabbit eye, measurement of induced glaucoma and stability evaluation as per modified ICH Q1A(R) guidelines for the drug product.

MATERIALS & METHODS:

The following materials are used for the study. Brimonidine Tartarate (FDC Limited Mumbai), Gelrite (Applied biosciences (KELCO).Mumbai), sterculia foetida gum (YUCCA enterprise, Mumbai). Kappa Carragennan (Gurukrupa industries Ahmedabad) Hydroxyl propyl methyl cellulose E50 LV (LOBA chemicals, Mumbai) All other chemicals were of analytical grade. Scitoz tonometer: Vision9, Bangalore. Proparacaine (Sunway PVT (L), Mumbai)ine Tartarate eye drops: (Allergan, India, Mumbai),5% Dextrose: (Fresenlus Kabi, Goa)2% Lignocaine hydrochloride:(Neon laboratories, Mumbai), High performance liquid chromatography:Model:2010A Shimadzu Japan, Stability chamber: Thermolab humidity chamber. India

Animals:

With the approval of Institute Animal Ethical Committee (IAEC/ABMRCP/PR/2012-2013/19), the study was performed and the protocol was approved as per CPCSEA guidelines. Albino rabbit (New zeland white rabbit) were used as test species. The right eye was designated as control and left one as test eye. In the lower conjunctival cul-de-sac, two drops of the formulation were instilled and for few seconds after instillation, eyelids were held together, later normal blinking was allowed.

Procedure for preparation of *in-situ* gels:

Added required quantity of gelrite polymer to the borate buffer solution and heated to about 70 ^{0}C until it is completely dissolved. To prepared gelrite solution required quantity of gum was added and stirred well on a magnetic stirrer with slight heating. To the above prepared gelrite/mucoadhesive solution, required quantity of drug (0.2% Brimonidine Tartarate) for their respective batches was added with continuous stirring until it is thoroughly mixed. hydroxy propyl methyl cellulose E50 LV and phenyl

ethyl alcohol were added and stirred on magnetic stirrer. pH was checked and adjusted with the buffer. The prepared *in- situ* gel were filled in glass vials and closed with closures, capped with aluminum caps and sterilized by autoclaving.

Experimental Animals: *In vivo* IOP lowering activity of *in-situ* gels of Brimonidine Tartarate was studied in normotensive albino rabbits of either sex, weighing 1.2-2.5 kg. [6]

Inducing of glaucoma by Marginal ear vein model
The rabbits were place in rabbit holders and left to stay for some time in order to de-stress. Hairs are plucked with help of scissor from the area of vein and a disinfected is applied. For the vein to be apparent the area is rubbed with alcohol or the vein is gently pressed on the base of ear vein or by padding a hot cloth on the ear in order to dilate the blood vessels. 2% lignocaine gel as local anesthetic was applied to the left ear and it was left for 30 minutes in order to desensitize it. A 24 gauge sterile butterfly syringe was inserted carefully in to the marginal ear vein and it was secured to ear with help of bandage. According to the body weight of the rabbits, 15ml/kg body weight, 5% dextrose solution was slowly and carefully avoiding air bubble it was infused into the ear vein with the help of 10ml syringe. After the solution has been injected the needle is slowly withdrawn, alcohol swab and pressure is applied to the puncture site for a short time in order to prevent bleeding. [7-11]

Estimation of IOP: Animals were appropriately restrained and IOP was estimated using calibrated Schiotz tonometer. Topical proparacain (0.5%) was used to produce corneal anesthesia before recording IOP. Three trials of each reading were carried out. Prior to inducing glaucoma /instilling the eye drops. IOP was measured with the help of Schiotz tonometer, which served as baseline value and then at 0, 30, 60, and 90 minutes thereafter at every 30 minutes interval till the baseline values were obtained. Time taken for the recovery of IOP were noted and compared in all and among the groups[12,13]

Study Drugs: Topical proparacaine (0.5%) for corneal anesthesia before recording IOP. Brimonidine Tartarate (0.2%) *in-situ* gels were used as Test. 0.2% Brimonidine Tartarate eye drops was used as a reference standard.

Experimental Protocol: For marginal ear vein model

Three normotensive rabbits were taken for each group.
1**.** Group I: - Normal IOP of rabbits was checked in both eyes of the rabbits. Three trials of each reading were carried out.
2. Group II: - glaucoma was induced to the rabbits. IOP was measured in both the induced eyes
3. Group III: - Two drops of Brimonidine Tartarate 0.2% (Formulation F2) sol-gel were topically instilled on to the cul de sac of left eye (test eye) and 2 drops of distilled water on to the right eye (control eye).

4. Group IV:- Two drops of Brimonidine Tartarate 0.2% (Formulation F1) sol-gel were topically instilled on to the cul de sac of left eye (test eye) and 2 drops of distilled water on to the right eye (control eye).

5. Group V:- Two drops of Brimonidine Tartarate (0.2%) eye drops were topically instilled on to the cul de sac of left eye (test eye) and 2 drops of distilled water on to the right eye (control eye).

Stability Study Testing Plan:
Batches: 0.2% Brimonidine Tartarate *in-situ* gel
Containers and closures: amber color glass vial with rubber closure and aluminum cap

Orientation of storage of containers: upright position
Sampling plan: 6 months for long term and for accelerated studies

Sampling time points: 0, 3, 6 for long term and 0, 1, 3, 6 months for accelerated studies
Number of testing: 3 times

Test storage conditions: long term 30^{o}C/75% RH and accelerated 40^{o}C/75% RH are used for the study.

Test parameters: visual appearance, pH, gelling capacity, sterility test, drug content

Test methodology: gelling capacity by gelation time, sterility test by direct transfer method, drug content by RP-HPLC method.

Acceptance criteria: pH (6.9-7.4), gelling capacity (++ = Immediate gelation, gel is stable, The vehicle is in the liquid form), sterility test (no growth was observed), % drug content (not less 5% of label claimed) Statistical evaluation of stability data by sigma plot 13 version software.

Optimized sterile formulation was subjected to stability studies as per modified ICH Q1A(R)

guidelines on stability testing of formulations (F2) Sterile optimized ophthalmic formulation was filled in glass vials, closed with butyl rubber closures and sealed with an aluminum caps. The vials were kept in the stability chamber, maintained at 30ºC/75% RH and 40ºC/75% RH for 6 months. Samples were withdrawn at 3, 6 for long term and 1, 3, 6 months for accelerated and were estimated for drug content, pH, visual appearance, gelling capacity, sterility test.

Stability study test parameters:

pH: The pH of the prepared *in-situ* gelling system was measured using pH meter.

Optical Clarity studies: Optical clarity of solutions/gels was carried out by using UV Visible Spectrophotometer (Shimadzu, 1700 Japan) against simulated tear fluid (7.4) as reference. Formulation was placed in a glass cuvette containing simulated tear fluid, care was taken to avoid air bubbles and the cuvette was inverted up and down to confirm gel formation. Transmission of light was measured at 580nm and it was kept constant for all batches. [14]

***In-vitro* gelation study**

The gelling time of formulation of different batches were determined by placing 1 or 2 drops of polymeric solution in a vial containing 2ml of freshly prepared simulated tear fluid (7.4 pH) equilibrated at 37°C. The gel formation was visually observed and time for gelation as well as time taken for the gel formed to dissolve was noted.[15]

Test for Sterility: Method of Direct Transfer

Tests for sterility were performed for fungi, aerobic and anaerobic bacteria using Soyabean Casein Digest media and Fluid Thioglycollate media.

Growth promotion (positive control) test: One culture tube containing 10ml of sterile media was inoculated with sterile loop full of micro-organisms and incubated as per the specified conditions. It is labeled as 'positive control'.

Sterility (negative control) test: Uninoculated sterile culture tube containing 10ml each for Fluid thioglycollate media and one for Soya bean Casein Digest medium were taken. These were incubated as per the specified conditions. It is labeled as 'negative control'.

Test for aerobic and anaerobic bacteria: Two culture tubes containing 10 ml each of sterile fluid thioglycollate media were labeled. 1 ml of the formulation was introduced to depth of culture tube with help of sterile syringe aseptically and labeled as depth D* (for anaerobic). To another culture tube of sterile fluid thioglycollate media, 1 ml of the formulation was introduced on to the surface of the culture media with help of sterile syringe aseptically. The tube labeled as surface S *(for aerobic). The four tubes (positive, negative and two labeled test tubes) were incubated at 35ºC for 14 days.

Test for fungi: Three sterilized culture tubes containing 10 ml each of sterile soybean-casein digest media were taken. The tube labeled as positive control was inoculated with sterile loop full of viable microorganism, Candida albicans aseptically. Uninculated culture tube was labeled as negative control. 1ml of the formulation was added to the culture tube aseptically and labeled as test. Three tubes were incubated at 25°C for 14 days. [16]

Analysis of Brimonidine Tartarate by RP- HPLC method

Chromatographic system

The chromatographic column used was C-18 (250mm×4.6mm) column with 5 µm particles. The mobile phase consists of Phosphate buffer (pH 3.0) : Methanol. The flow rate of the mobile phase was kept at 0.8 ml/min and the column temperature was maintained at 35^0C and the chromatogram was monitored at a wavelength of 248 nm. The injection volume was 10 µl with pump pressure of 13.0Mpa

Preparation of mobile phase

Dissolved 1.02 g of Potassium dihydrogen ortho phosphate in 500ml of water. Mixed the contents to dissolve. Then it was sonicated and filtered through 0.45 µ filter. The pH was adjusted to 3.0 with ortho phosphoric acid, and then Buffer (pH 3) and Methanol were mixed in the ratio of 85:15.

Drug content by RP- High performance liquid chromatography:

The High performance liquid chromatography was stabilized, the mobile phases were selected and the column was auto purged. Later the column was stabilized with the mobile phase with its different ratio, wave length and pump pressure. Blank mobile phase chromatogram was run. 0.1ml of sample was diluted to 10ml and transferred in to the vials. The vials were placed in the trays and chromatogram was run by using shimadzu LC Solution software. The obtained chromatogram was manually integrated with help of software. False peaks were rejected and the area of the peak was marked. In the data acquisition step, the results were obtained as retention time, area height peak start time and peak end and tailing factor. The stability data was analyzed statistically by using

sigma plot version 13 with 95 % confidence interval [17.]

RESULTS:

Table No 1: Composition of Various Formulations

Formulation code	Ingredients %						
	Brimonidine Tartarate	Gelrite	Kappa carrageenan	Sterculia foetida gum	HPMC E 50LV	Phenyl ethyl alcohol	Borate buffer Q.S
F1	0.2	0.39	0.21	--	0.4	0.5	100ml
F2	0.2	0.24	--	0.13	0.4	0.5	100ml

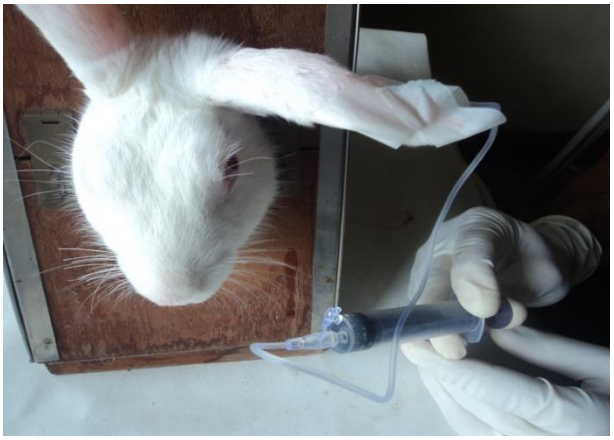

Fig. No. 1: Infusion of 5% dextrose solution through marginal ear vein

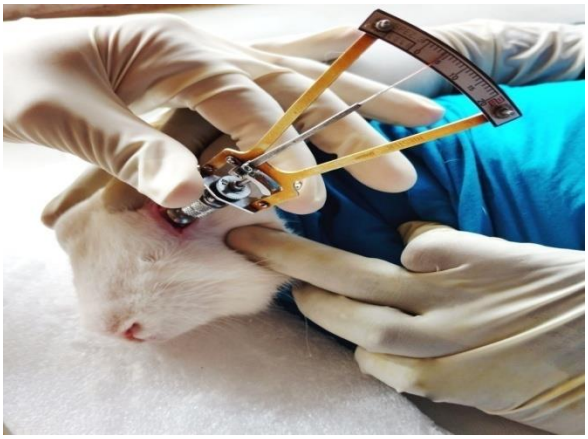

Fig. No. 2: Measurement of IOP by Schiotz Tonometer

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Statistical methods used for analysis:-

Research question:- *In-situ* gel are better in controlling induced intra ocular pressure compare to eye drops.

Null hypothesis (H_0):- $H_0 = Ha$

Alternative hypothesis (Research Hypothesis) :- $H_0 \neq Ha$

Significance level α: p-value < α = 0.05

If p-value < α = 0.05, If the *p*-value is less than 0.05. We reject the null hypothesis and support the alternate hypothesis.

If p-value > α = 0.05, If the *p*-value is more than 0.05. We fail to reject the null and do not support the alternate hypothesis

Case:-I (Normal IOP vs. Induced IOP)

Null hypothesis (H_0):- Glaucoma is not induced by hypotonic agent

Alternative hypothesis (Ha):- hypotonic agent induces glaucoma by reducing blood osmolarity.
Case:-II .A. (Induced IOP vs. Induced treated *in-situ* gel) (Dependent variable / student T-test)
Null hypothesis (H_0):- *In-situ* gel is not able to control the induced IOP
Alternative hypothesis (Ha):- *In-situ* gel is able to control the induced IOP
Case:-II.B. (Induced IOP vs. Induced treated *in-situ* gel) (Independent variable /ANOVA)
Null hypothesis (H_0):- *In-situ* gel is able to control the induced IOP
Alternative hypothesis (Ha):- *In-situ* gel is not able to control the induced IOP
Case:-III (Induced treated in-situ gel) vs (Induced Standard eye drops)
Null hypothesis (H_0):- *In-situ* gel is not effective in sustaining anti glaucoma activity for longer duration
Alternative hypothesis (Ha):- *In-situ* gel is effective in sustaining anti glaucoma activity for longer duration

Table No 2: Intra-ocular pressure of various groups in mm Hg.

Time in minutes	Group no I normotensive		Group no II glaucoma induced		Group no III (Test F2)		Group no IV (Test F1)		Group no V (Standard)	
	Left Eye Mean±SEM	**Right Eye** Mean±SEM	**Left Eye** Mean±SEM	**Right Eye** Mean±SEM	**Left Eye** (F2) Mean±SEM	**Right Eye (Distilled Water)** Mean±SEM	**Left Eye** (F1) Mean±SEM	**Right Eye (Distilled Water)** Mean±SEM	**Left Eye** (F1) Mean±SEM	**Right Eye (Distilled Water)** Mean±SEM
0	17.66±0.63	17.50±0.00	18.4±0.63	18.7±0.36	17.73±0.33	18.10±0.90	18.03±0.36	18.74±0.72	18.40±0.00	18.40±1.10
30	18.83±1.15	18.10±0.30	27.4±0.00	26.0±0.80	22.45±1.53	26.01±0.80	19.50±0.00	30.83±1.05	18.40±0.63	27.55±0.81
60	18.40±0.00	17.50±1.15	30.2±0.61	28.4±0.51	21.44±0.42	27.46±0.88	23.13±0.00	31.44±0.64	18.40±0.12	28.58±1.42
90	17.36±0.11	16.83±0.66	28.4±0.51	30.2±0.61	21.88±0.02	29.08±0.96	25.93±0.00	29.10±0.97	21.64±2.14	29.10±0.97
120	18.10±0.52	17.80±0.30	29.7±0.56	30.2±0.61	20.28±0.78	30.22±0.61	24.91±0.51	27.56±0.97	27.55±1.44	28.58±1.43
150	19.56±1.79	18.76±0.36	29.7±1.12	28.4±0.51	19.92±1.02	29.61±0.61	25.42±0.51	27.98±0.51	27.56±0.97	27.46±0.88
180	17.06±0.40	18.83±0.33	28.4±0.51	27.9±1.02	20.28±0.78	28.48±0.51	24.06±0.93	26.95±0.51	27.65±0.90	26.53±0.47
210	18.16±2.30	17.86±0.36	26.9±0.51	26.9±0.51	19.86±0.36	27.46±0.00	24.06±0.93	25.93±0.00	27.46±0.03	27.97±0.51
240	18.83±1.15	17.50±1.15	25.0±1.61	25.5±0.42	18.76±0.36	24.57±0.80	23.64±1.20	25.42±0.51	25.08±0.42	25.51±1.26

S.D*=Standard Deviation (n=3), SEM= Standard Error of Mean

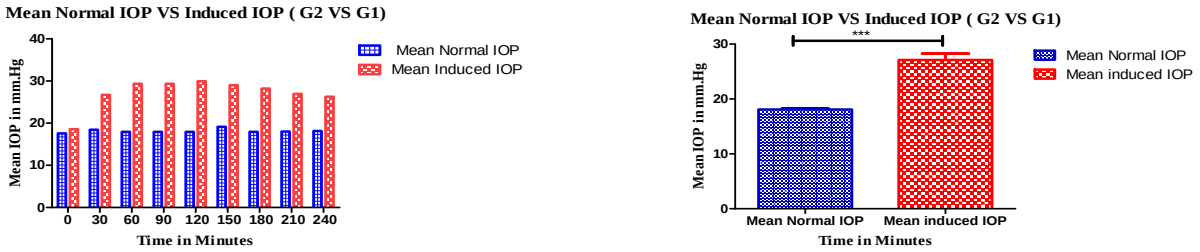

Fig.No: 3. Normal IOP vs Induced IOP (Group No I vs Group No II).

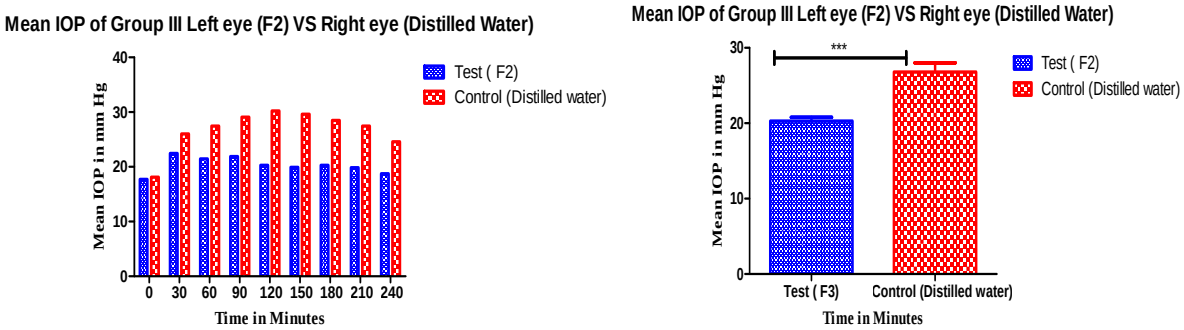

Fig.No 4. Mean IOP of group III LE (F2) vs RE (Distilled water)

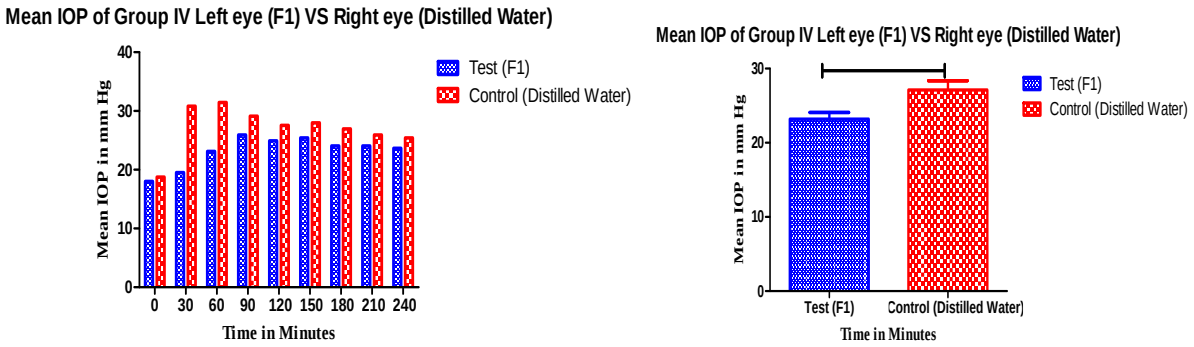

Fig.No 5.mean IOP of group IV LE (F1) vs RE (Distilled water)

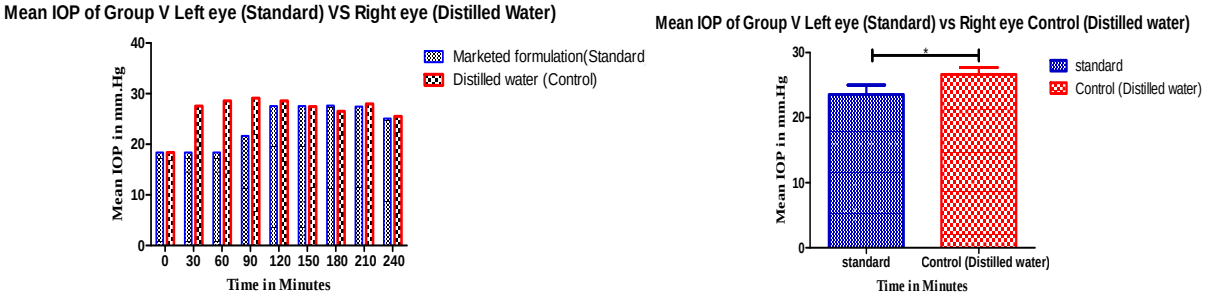

Fig.No: 6. Mean IOP of group V left eye with Standard vs Right eye (Distilled water)

Table No 3: Statistical data of Group III (Test) vs GroupV (Standard) rabbits

Group III (Test) vs GroupV(Standard)	Tabular results	Group III (Test) vs GroupV(Standard)	Tabular results
Un-Paired t test		Paired t test	
P value	<0.0235	P value	<0.0377
P value summary	**	P value summary	*
Are means signif. different? (P < 0.05)	**Yes**	**Are means signif. different? (P < 0.05)**	**Yes**
One- or two-tailed P value?	One-tailed	One- or two-tailed P value?	one-tailed
t, df	t=2.152 df=16	t, df	t=2.043 df=8
How big is the difference?		How big is the difference?	
Mean ± SEM of Normal IOP	20.29 ± 0.4963	Mean ± SEM of Normal IOP	-3.060
Mean ± SEM of Induced IOP	23.57 ± 1.442	Mean ± SEM of Induced IOP	-6.514 to 0.3937
Difference between means	-3.282 ± 1.525	Difference between means	0.3429
95% confidence interval	-6.515 to -0.04904	95% confidence interval	
R squared	0.2245	R squared	0.3271
F test to compare variances		F test to compare variances	0.1951
F,DFn, Dfd	8.444, 8, 8	F,DFn, Dfd	*
P value	< 0.0068	P value	Yes
P value summary	**	P value summary	<0.0377
Are variances significantly different?	Yes	Are variances significantly different?	*

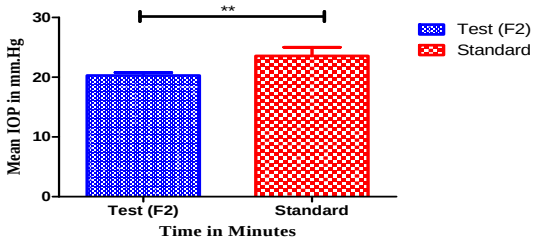

Fig.No 7. Mean IOP of Group III (Test) vs Group V (Standard) rabbits

Table No 4: Repeated Measures one –way ANOVA with Tukey's Multiple Comparison Test(F2 & F1)

Repeated Measures one –way ANOVA with Tukey's Multiple Comparison Test					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	5				
F	17.42				
R squared	0.6853				
ANOVA Table	SS	df	MS		
Treatment (between columns)	373.4	4	93.35		
Individual (between rows)	147.0	8	18.38		
Residual (random)	171.4	32	5.358		
Total	691.9	44			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Normal IOP vs Induced IOP	-8.282	9.735	Yes	***	-11.60 to -4.964
Normal IOP vs Test (F2)	-1.360	1.599	No	ns	-4.679 to 1.959
Normal IOP vs Standard	-4.730	5.560	Yes	**	-8.049 to -1.411
Induced IOP vs Test (F2)	6.922	8.137	Yes	***	3.604 to 10.24
Normal IOP vs F1	-4.218	5.467	Yes	**	-7.373 to -1.063
Induced IOP vs F1	4.064	5.268	Yes	**	0.9092 to 7.220
Induced IOP vs Standard	3.552	4.176	Yes	*	0.2335 to 6.871
Test (F2) vs Standard	-3.370	3.961	Yes	*	-6.689 to -0.05132
Test (F2) vs F1	-2.858	3.704	Yes	**	-6.013 to 0.2975
Standard vs F1	0.5122	0.6639	No	ns	-2.643 to 3.667

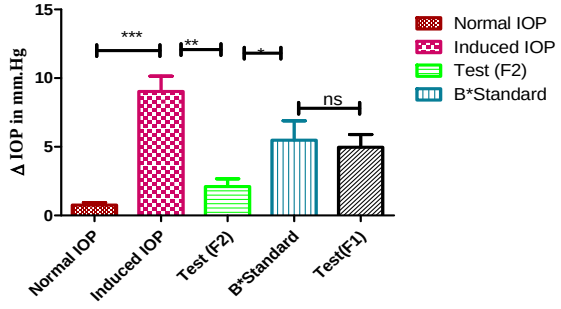

Fig.No 8. Repeated Measures one –way ANOVA with Tukey's Multiple Comparison Test

Table No 5: Hypothesis testing of Group No I to Group No V of rabbits (Left eye) in mm Hg.

Groups	Reject the Null hypothesis(R*)	Support the Alternative hypothesis(S*)	Fail to reject the null hypothesis(FR*)	Case
Group No II	R	S	--	Case -I
Group No III:	R	S	--	Case –II.A
		NS*	FR	Case –II.B
Group III vs Group V	R	S	-	Case-III
Group IV vs Group V	--	NS	FR	Case-III
Group III vs Group IV	R	S	--	Case-III

NS*= Not support

Table No 6: Measurement of IOP In mm Hg at 120 th minute (Marginal ear vein method).

Measurement of IOP In mm Hg at 120 th minute (**Marginal ear vein method)**		
Group	Left Eye (Treated*)	Right Eye (control**)
Normal IOP	18.10±0.52	17.80±0.30
Induced IOP	29.70±0.56	30.20±0.61
Sterculia Foetida (F2)	20.28±0.78	30.22±0.61
Kappa Caragennan (F1)	24.91±0.51	27.56±0.97
Brimonidine Tartarate Marketed eye drops	27.55±1.44	28.58±1.43

Table No 7: Long term studies at different months (0, 3, 6)

Long term studies of F2 Formulation at 30 °C ± 2 °C/65% RH ± 5% RH different months						
	Visual Appearance	pH	Gelling Capacity	Sterility Test		%Drug Content±S.D*
				Bacterial media	Fungal media	
0 month	Translucent	7.39±0.03	++	No growth was observed	No growth was observed	99.35±0.65
3 month	Translucent	7.30±0.05	++	No growth was observed	No growth was observed	99.25±1.55
6 month	Translucent	7.14±0.09	++	No growth was observed	No growth was observed	98.66±1.74

- (++) Immediate gelation, gel is stable, The vehicle is in a liquid form

Table No 8: Accelerated stability studies at different months (0,1, 3, 6)

Accelerated stability studies of F2 Formulation at 40°C ± 2°C/75% RH ± 5% RH for different months						
	Visual Appearance	pH	Gelling Capacity	Sterility Test		%Drug Content±S.D*
				Bacterial media	Fungal media	
0 month	Translucent	7.40±0.02	++	No growth was observed	No growth was observed	99.46±0.204
1 month	Translucent	7.36±0.04	++	No growth was observed	No growth was observed	98.32±0.408
3 month	Translucent	7.26±0.07	++	No growth was observed	No growth was observed	97.81±1.058
6 month	Translucent	7.13±0.24	++	No growth was observed	No growth was observed	95.61±1.722

*Standard Deviation (n=3)

System suitability studies of Brimonidine Tartarate by RP-HPLC method

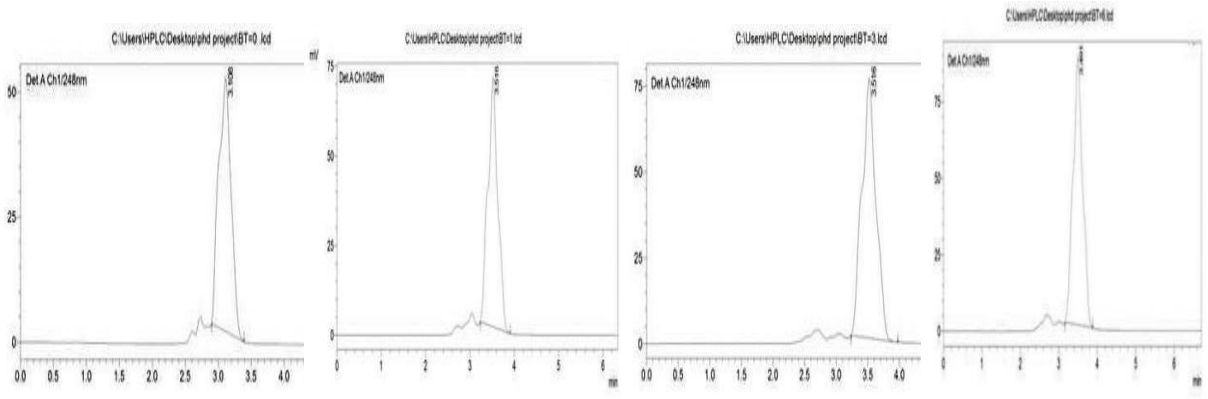

Fig No: 9.a. At 0 month Fig No: 9.b. At 1 month Fig No: 9.c. At 3 month Fig No: 9d. At 6 month

Fig No: 9.chromatograms of Brimonidine Tartarate(F2) for different months at40°C ± 2°C/75% RH ± 5% RH

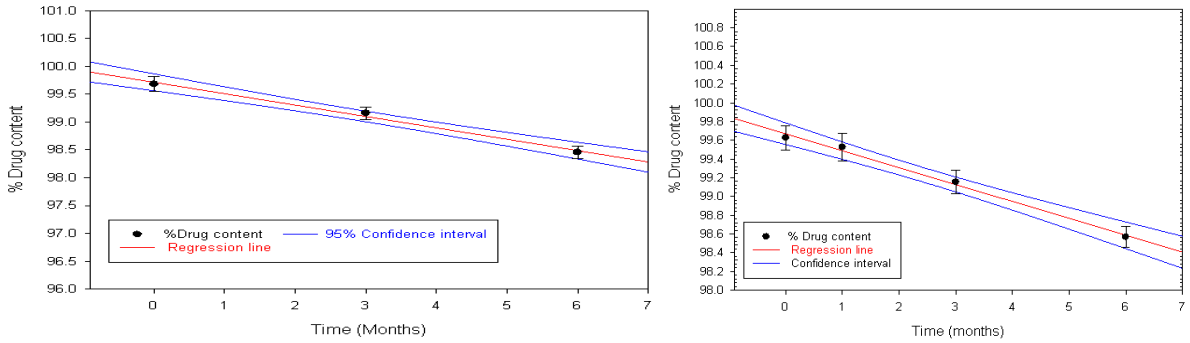

Fig No: 10: Regression control chart: percentage drug content of Brimonidine Tartarate (F2) at (0,3,6) months with 95% confidence interval at 30 °C ± 2 °C/65% RH ± 5% RH and at 40°C ± 2°C/75% RH ± 5% RH (0,1,3,6) months.

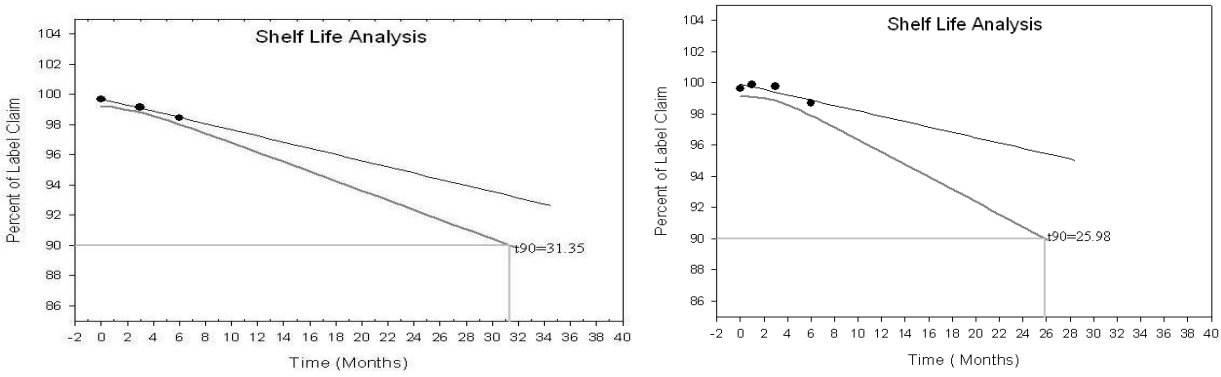

Fig No: 11: shelf life analysis of Brimonidine Tartarate (F2) at (0,3,6) months with 95% confidence interval at 30 °C ± 2 °C/65% RH ± 5% RH and at 40°C ± 2°C/75% RH ± 5% RH (0,1,3,6) months.

Table no: 10 shelf life of Brimonidine Tartarate (F2) at different temperature and relative humidity

Sl.no	Sample No	Shelf life in months	
		30 °C ± 2 °C/65% RH ± 5% RH	40°C ± 2°C/75% RH ± 5% RH
1	F2	31.00	25.00

DISCUSSION:

Animal model:- Elevation of intraocular pressure (IOP) is a critical risk factor for glaucoma progression, and its lowering has become a major focus of intervention. Animal studies have articulated the mechanisms of the formation and evacuation of aqueous humor as well as the maintenance of intra-ocular pressure, thereby informing glaucoma etiology and therapeutic development.

Study design: - 5 % dextrose infusion through marginal ear vein forms the easiest, fastest and reliable techniques to screen anti-glaucoma agents. 5 % dextrose infusion leads to reduction in the blood osmolality, which leads to transfer of water in to the eye, causing elevation of IOP. Choroid is the body tissue with highest blood supply per area, about 70-80% of ocular blood flow corresponds to choroidal vasculture. Ingestion of hypotonic fluids leads to its absorption from different body tissues. However, once the dextrose has been absorbed by the body, then only plain water is left in the intravascular space. And plain water is clearly hypotonic, intravenously administered dextrose lowers serum osmolarity. This reduced serum osmolarity leads to the movement of water into the eye thereby increasing IOP. Schiotz tonometer remains the preferred screening instrument. Accuracy problems assume a lesser importance when compared to the difficulty in knowing the exact relationship between elevated intraocular pressure and the development of glaucoma. Schiotz tonometer is capable of providing measurements accurate enough to screen for a disease that has a long latency period before producing symptoms. The instrument is relatively inexpensive, possible to work with a minimum of effort, and it is acceptable by most patients.

Group No I: The mean normotensive IOP was in the range of 17.5±0.08 to 19.1±0.40 mm Hg in both the eyes.

Group No II: Glaucoma was induced in the both eye with 5% dextrose solution and the mean IOP in both eyes was in the range of 26.2±0.21 to 29.9±0.25 mm Hg. Student unpaired T-test (G. I (Normal) vs. G. II (Induced) was analysed and it was found that there is a highly significant difference between the mean. The p value was 0.001. The repeated measures one –way anova with tukey's multiple comparison test also indicate the data is highly significant. This clearly indicates that glaucoma is induced. So we reject the null hypothesis and support the alternative hypothesis of case-I. Table No 2,4,5

Group No III:

- Brimonidine tartarate *in-situ* gel with sterculia foetida gum (F2)

Brimonidine taratarate *in-situ* gel with sterculia foetida gum (F2) was instilled in the induced left eye and distilled water in the induced right eye. The mean IOP of left eye was found to in the range of 18.76±0.36 to 22.45±1.53. Where as in the right eye it was found in the range of 24.57±0.80 to 30.22±0.61. Student paired T-test (G. III (F2*) vs. G. III(Distilled water) was analyzed and it was found that there is a highly significant difference between the means. The p value was 0.002. This indicates the test drug is effective in the form of *in-situ* gels. So we reject the null hypothesis and support the alternative hypothesis of case –II.A. (i.e *In-situ* gel are able to control the induced IOP and the IOP values of treated *in-situ gel* are near to normal IOP reading). The repeated measures one –way anova with tukey's multiple comparison test indicate the data is not significant, i.e p value is greater than p<0.05. so we fail to reject null hypothesis and cannot support alternative hypothesis of case –II.B.(i.e *In-situ* gel are able to control the induced IOP and the IOP values of treated *in-situ* gel are near to normal IOP reading). Fig No 4 Table No 5

Group III vs Group IV:

- Brimonidine taratarate *in-situ* gel with sterculia foetida gum (F2) vs Brimonidine tartarate *in-situ* gel with Kappa carragennan (F1).

The mean IOP (F1) of left eye was found to be in the range of 19.50±0.00 to 25.93±0.00 and that of right eye with distilled water it was found to be in the range of 25.42±0.51 to 30.83±1.05 and F2 it was found to be in the range of 18.76±0.36 to 22.45±1.53 and that of right eye with distilled water it was found in the range of 24.57±0.80 to 30.22±061. Student unpaired and paired T test along with one way ANOVA data shows that there is a marginal significant difference between the means of F1 and F3. With p value of <0.0060 for student unpaired, <0.0050 for student paired T test. There is no significant difference between F3 and normal IOP but

there is a marginal significant difference between F1 and normal IOP. Which highlight F3 is better in controlling induced/elevated IOP compared to F1. Fig No 5

Group III vs Group V:

- (F2) vs Brimonidine tartarate (B*) eye drops)

The mean IOP of marketed (B*) eye drops was found to be in the range of 18.40±0.12 for the first one hour later increased to 27.65±0.90 (21.64±2.14 to 27.46±0.03) for next two and half hour. The mean IOP of induced right eye was found to be in the range of 25.51±1.26 to 29.10±0.97 throughout the four hour study. Student paired T test that there a significant difference between the means of induced treated with standard eye drops along and induced control eye along with a p value of 0.0377. Student unpaired T test and one way ANOVA data shows that there is a marginal significant difference between the means of F2 and standard eye drops with a p value of 0.0235. Since the p value is less than 0.05, the null hypothesis is rejected and alternative hypothesis is supported as in Case-III.(i.e *In-situ* gel are able to sustain the anti glaucoma activity for 4 hours and it IOP values are near to normal IOP reading). Table No.3,5,8 & Fig No 7

Group IV vs Group V:

- (F1) vs Brimonidine tartarate (B*) eye drops)

Student paired T test showed that there a significant difference between the means of induced treated with standard eye drops along and induced control eye along with a p value of 0.005. Student unpaired T test and One way ANOVA data showed there is a no significant difference between the means of F1 and standard eye drops with a p value of 0.4118. Since the p value is more than 0.05, the null hypothesis is fail to rejected and alternative hypothesis is not supported as in Case-III.(i.e *In-situ* gel are not effective to sustain the anti glaucoma activity for 4 hours and it IOP values are near to induced standard eye drops). F1 is effective to sustain induced IOP with values marginal lesser than control induced IOP but are not near to normal IOP.

pH study: The pH of formulation F2was (7.40-7.22) at 30 °C ± 2 °C/65% RH ± 5% RH and (7.40-7.21) at 40°C ± 2°C/75% RH ± 5% RH Table no.7,8

Optical Clarity studies: Gels with optical transmission≥90% are classified as transparent, ≤ 90% but ≥10% as translucent, and ≤ 10% as opaque. The study revealed that *in-situ* gels were translucent at 30 °C ± 2 °C/65% RH ± 5% RH and at 40°C ± 2°C/75% RH ± 5% RH. Table no.7,8

In-vitro gelation study: Optimum gelling was obtained from the both F2 when stored at different temperatures and at various interval of time. Immediate gelation was formed with in sec, as it was dropped and remained stable at 30 °C ± 2 °C/65% RH ± 5% RH and at 40°C ± 2°C/75% RH ± 5% RH. Table no.7,8

Test for Sterility: Sterility test: Sterility test of a product is the absence of viable and actively multiplying micro-organisms when tested in specified culture media. The formulation incubated with media suitable for the growth and proliferation of aerobic/ anaerobic bacteria, fungi showed no growth at the end of 14 days at 35 °C and at 25 °C. No evidence of microbial growth/ turbidity was found in the test and negative samples when compared with positive control media. This indicated that formulations were free from micro-organisms at 30 °C ± 2 °C/65% RH ± 5% RH and at 40°C ± 2°C/75% RH ± 5% RH. So the preparations being examined comply with the test for sterility. Table no.7,8

Chromatographic studies: Chromatograms of Brimonidine Tartarate in Phosphate buffer (pH 3) and Methanol (85:15 *% v/v)*.The calibration curves and regression equation for Brimonidine Tartarate was within range.The LOD and LOQ of Brimonidine Tartarate were found to be 0.075 µgmL−1 and 0.227 µgmL−1, respectively which show that the method is specific. The values obtained for system suitability parameters were found to be within the limits.

Drug content by RP-HPLC: the samples were analyzed for drug content by HPLC Method. The drug degraded to a negligible extent, and the percentage of drug degradation is <5% .The chromatograms were obtained with acceptable tailing factors (<2), not much variation was observed in the retention time. % drug content was found to be in the range of 99.46 % to 96% at 40°C ± 2°C/75% RH ± 5% RH by RP-HPLC. Table No.7,8,9. Fig No.9(a-d).

Shelf life: The shelf life (t90%) of F3 & F7 was found to be 2.5 years at 30 °C ± 2 °C/65% RH ± 5% RH . 40°C ± 2°C/75% RH ± 5% RH shelf life (t90%) was found to be 1.5 to 2 years. Table No.10. Fig No.10,11.

CONCLUSION:

Rabbits share many common traits with humans, including similar physiology and heterogeneous genetic background. Indeed, phylogenetically rabbits are closer to primates than to rodents. Rabbits highly suitable for testing safety and efficacy of novel approaches for treatment of ocular diseases. 5 % dextrose infusion through marginal ear vein forms the easiest, fastest and reliable techniques to screen anti-

glaucoma agents. Schiotz tonometer remains the preferred screening instrument. The instrument is relatively inexpensive, possible to work with a minimum of effort, and it is acceptable by most patients.

The induce IOP is maintained for longer period (4hours) in marginal ear vein method Marginal ear model is better in inducing IOP. Brimonidine Tartarate has a dual *mechanism of action* by reducing aqueous humor production and increasing uveoscleral outflow. So a *synergistic effect can be obtained with help of in-situ gels in order to control elevated intra-ocular pressure in glaucoma.* The above study highlights that formulation with sterculia foetida is better compared to kappa carragennan in controlling induced IOP.

The stability studies are essential for well being of the patient suffering from disease for which product is designed. Degradation of unstable product (drug) into decomposition product yield toxic by product which is harmful to patient. loss of activity up to a level of 85% of the claimed on the label may lead to failure of the therapy resulting in death it has become a legal requirement to provide data on stability studies according to the guidelines so that recommended storage conditions and shelf life can be included on the label to ensure that the medicine is safe and effective throughout its shelf life.

pH of formulations F2 are within the range of comfort (6.8 to7.8). Hence formulations can be well tolerated by the eyes. Gelling was immediate and remained stable. The formulations were in liquid form and will form gel on contact with tear fluid, with no drainage from the eye. Sterility test at interval during incubation period, and at its conclusion, the test and negatives medias showed no sign of microbial growth. Thus the preparation being examined passes the test for sterility. The drug content showed the uniform dispersion of the drug throughout the formulation. It also highlights that, there is no interaction between the drug and polymers. From stability study it reveals that formulation F2 showed no change in physical appearance and formulation showed very less decrease in drug content, percentage of drug degradation is <5% . From this study it reveals that tested formulation were stable. Since the overall degradation is <5%, a supported shelf life of 2 years may be assigned to the optimized formulation.

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