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Formulation and evaluation of acetazolamide loaded *in–situ* gel for the treatment of glaucoma

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ABSTRACT: The present research work was proposed to develop a pH triggered *in-situ* gel formulation for the ocular delivery of acetazolamide to treat glaucoma. Carbopol-934, Carbopol 940, Hydroxypropyl methylcellulose (HPMC) K4M, and Sodium Alginate were chosen for the development of *in-situ* gel. Formulation with Carbopol 940 and sodium alginate was optimized by assessing the gelling capacity. The prepared *in-situ* gel formulations were thoroughly characterized for gelling time, gelation temperature, Fourier-transform infrared (FTIR) spectroscopy studies, rheological study, sterility testing, corneal drug permeation, ocular irritation test, and accelerated stability study. Ex-vivo corneal permeation study was performed using goat cornea. The result of transcorneal permeation of acetazolamide followed the Fickian diffusion process. The optimized formulations showed satisfactory gelling time (4.17-5.17 sec) and dissolution time (120 min). FTIR study confirms the compatibility between the polymers and acetazolamide. The sterility study showed a satisfactory result. After 72 h observation in the *in-vivo* rabbit eye irritation study, the eyes appeared normal. There were no significant changes in pH and drug content in the accelerated stability studies of the formulations.

KEYWORDS: Acetazolamide; gelation time; carbopol-934&940; sodium alginate; *in-situ* gel; release; viscosity.

1. INTRODUCTION

The eye is a sense organ designed to respond to light to enable vision. The majority of the information we receive from our surroundings comes from vision, which has noteworthy physiological significance. This includes the ability to distinguish between different types of light, shapes, and colors, as well as spatial direction, equilibrium, and cortical tone [1]. The complicated anatomical structure and defensive mechanisms of the eyes make it difficult to maintain an effective drug concentration over an extended period of time, which makes the development of ocular drug delivery systems to be difficult [2-4]. Among all the conditions, Glaucoma is the world's most prevalent eye disease and the leading cause of permanent blindness [5]. It is characterized by 'tunnel vision,' which is caused by the purposeful degeneration of retinal ganglion cells. High intraocular pressure (IOP) is one of the most dangerous characteristics of glaucoma [6]. Glaucoma affects more than 67 million people globally, according to the World Health Organization [7]. Nearly 80 million individuals have open-angle and angle-closure glaucoma by the year 2020. Conventional dosage forms have a major drawback to provide prolonged and controlled release of drugs from the dosage form [8]. Nowadays researchers are focused on novel formulations like ocular-insert, and *in-situ* gel formulations to improve the efficacy and to minimize side effects. Lowering IOP is one of the best methods to reduce the *risk* of glaucomatous visual field loss [6].

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By lessening the impact of ocular drainage and/or flow on the cornea and lengthening corneal residence time, bioavailability may be improved. Different polymers have been employed to improve the viscosity of eye drops and the adhesion of solutions injected into the eye in order to prolong corneal residence duration [7]. *In-situ* drug delivery technology is one of them. The said technology has come under a novel drug delivery system in which polymeric or gum-based formulations are able to transit from solution to gel (sol-to-gel) phase in response to external stimuli including pH change, temperature change, presence of ion, solvent exchange, swelling diffusion, and chemical cross-linkage. The increased popularity of the sol-to-gel system is due to various advantages like ease of administration, decreased frequency of administration, prolonged drug release, and improved patient comfort [9].

Acetazolamide (ACZ), is a class IV drug as per the biopharmaceutical classification system (BCS) [10]. It is a carbonic anhydrase inhibitor (CAI), used to treat open-angle glaucoma's severe symptoms. It delays the onset of blindness and lowers IOP before surgery [11]. Among the currently available CAIs, ACZ continues to be the most popular and most efficient medication for the treatment of open-angle glaucoma. ACZ inhibits the carbonic anhydrase enzyme (CAE), which is important for the movement of carbon dioxide from the tissues to the lungs and for promoting the generation of aqueous humor [12]. However, with the widespread distribution of the CAE enzyme, large oral dosages of acetazolamide typically cause a wide range of systemic adverse effects in addition to lowering IOP [11].

The objective of the present study is to formulate ACZ loaded pH triggered in-*situ* gel delivery system for topical application to treat glaucoma.

2. RESULTS AND DISCUSSION

2.1. Calibration curve of ACZ

ACZ was taken and various concentrations of ACZ solution were prepared by using Simulated Tear Fluid (STF). Suitable dilution of drug solution were made and absorbance was taken using UV-Visible spectrometer at 267nm (λ max). A graph was plotted (Figure 1).

2.1.1. Drug-Excipient interaction study

The physical mixture of drug and polymer was prepared in 1:1 ratio. The powder mix was then dissolved in distilled water. The physical interaction was visually inspected for change in color, formation of bubbles, agglomeration, and change in odor. No physical interaction was observed in both the physical mixture and solution.

2.2. Optimized formulations

Three best formulations (F12, F13, and F14) were selected for further characterization. The formulations are selected owing to rapid gelling time and slow dissolution time (Table 2).

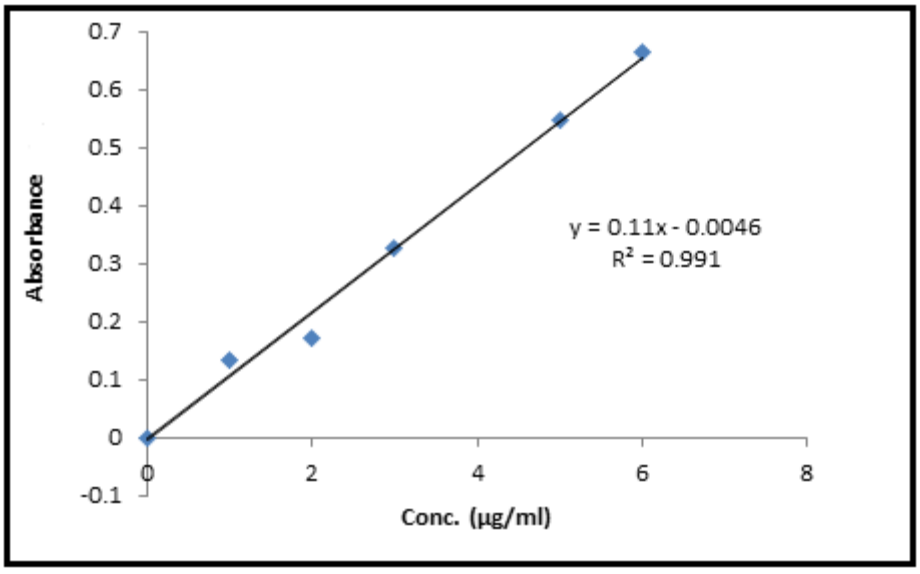

Figure 1. Calibration curve of ACZ

Table 2. Optimized formulations

Ingredients	Formulations		
	F12	F13	F14
Acetazolamide (%)	0.5	0.5	0.5
Carbopol-934 (%)	0.2	0.3	0.4
Sodium Alginate (%)	0.5	0.8	1.0
Benzalkonium chloride (%)	0.02	0.02	0.02
Distilled Water	100	100	100

2.2.1. Determination of gelling and dissolution time

The gelling time was studied by adding a drop of formulation into the specified volume of STF. The gelling time of F14 was lowest (4.17 ± 0.29 sec) followed by F13 (4.77 ± 0.67 sec) and F12 (5.17 ± 0.76 sec). The gelling time was found to be dependent on the concentration of carbopol 934 and sodium alginate in the formulation. The higher the concentration of the polymer showed the lower gelling time. However, no significant difference was observed in the dissolution time of all three optimized formulations. The dissolution time for F12, F13, and F14 were found as 120.0 ± 1.00, 120.0 ± 2.00, and 120.0 ± 1.00 min respectively. The result of the gelling time and the dissolution time was presented in figure (Figure 2).

2.2.2. Appearance, clarity, and pH

The appearance of the formulations was observed by visual inspection. The formulation F12 was transparent, whereas F13 and F14 were translucent. The clarity of the formulations was evaluated by placing the formulations in front of a white and black background. All formulations were clear. The pH of all the optimized formulations was in the range of 4.3 to 4.9 (Table 3). The appearance and clarity of the formulations remained unchanged after autoclaving.

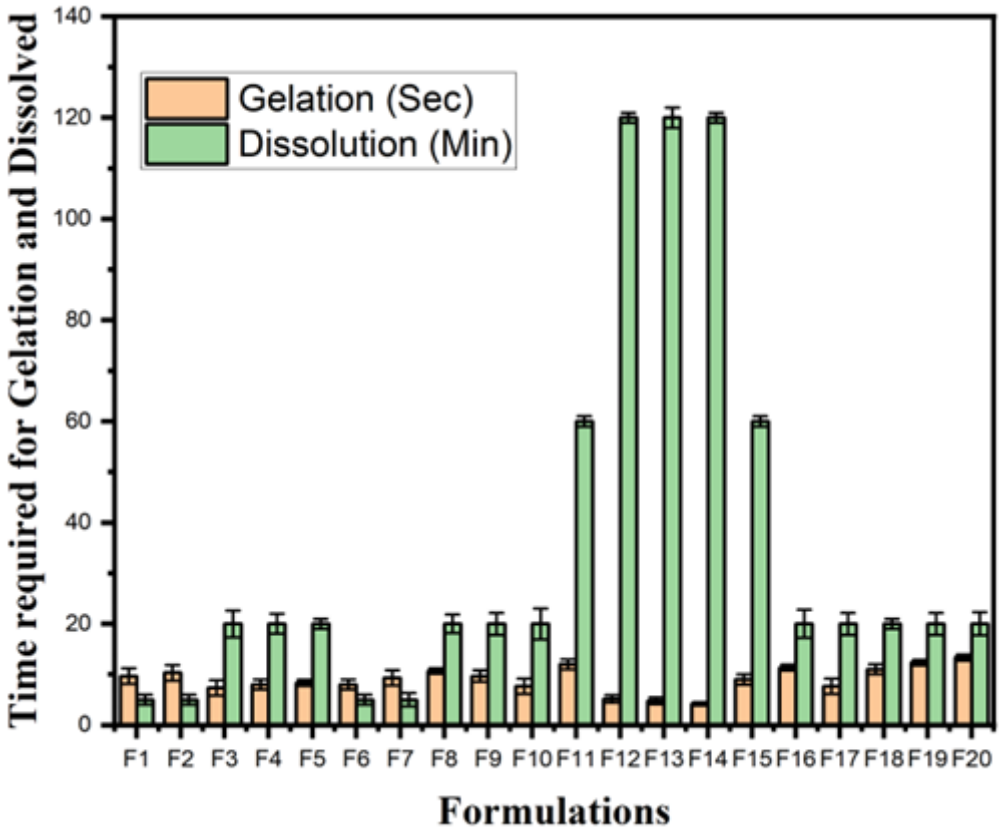

Figure 2. Gelation time vs dissolution time (n=3)

Table 3. Physical property of the optimized formulation

Formulations	Physical Parameters			
	Appearance	Clarity	pH	Gelation Temperature (°C)
F12	Transparent	Clear	4.9 ± 0.08	37.2 ± 0.2
F13	Translucent	Clear	4.5 ± 0.04	38.6 ± 0.6
F14	Translucent	Clear	4.3 ± 0.05	40.5 ± 0.3

2.2.3. Rheological study

The result of the viscosity of the optimized *in-situ* gel was shown in table and figure (Table 4 and Figure 3). The result showed an increase in viscosity of the formulations with the increase the pH. The pH of the formulations F12, F13, and F14 was 4.9, 4.5, and 4.3 respectively. The pH of the tear is ~7.2 [13]. Increase the pH to 7.2, the viscosity of F12, F13, and F14 was increased from 75 cPs to 100 cPs, 200 cPs to 350 cPs, and 475 cPs to 675 cPs, respectively. Thus, an increase the pH of all formulations to 7.2, an increased viscosity of 1.33 times, 1.75 times, and 1.42 times was observed in F12, F13, and F14, respectively. The sol-to-gel transformation of the formulation at ocular pH improves the ocular residence time and bioavailability.

2.2.4. Gelation temperature

Gelation temperature (GT) of the optimized formulation was shown in table (Table 3). The GT of F12 was 37.2 ± 0.2 °C followed by 38.6 ± 0.6°C (F13) and 40.5 ± 0.3°C (F14). The GT of the formulations was observed to be increased with the increase of the concentration of Carbopol 934 and sodium alginate.

Table 4. Viscosity of the *in-situ* gel.

Formulations	Before adjusting pH		After adjusting pH		Enhanced ratio
	pH	Viscosity (cPs)	pH	Viscosity (cPs)	
F12	4.9 ± 0.08	75 ± 1.73	7.2 ± 0.03	100 ± 9.16	1.33
F13	4.5 ± 0.04	200 ± 3.60	7.2 ± 0.03	350 ± 15.00	1.75
F14	4.3 ± 0.05	475 ± 3.00	7.2 ± 0.03	675 ± 13.22	1.42

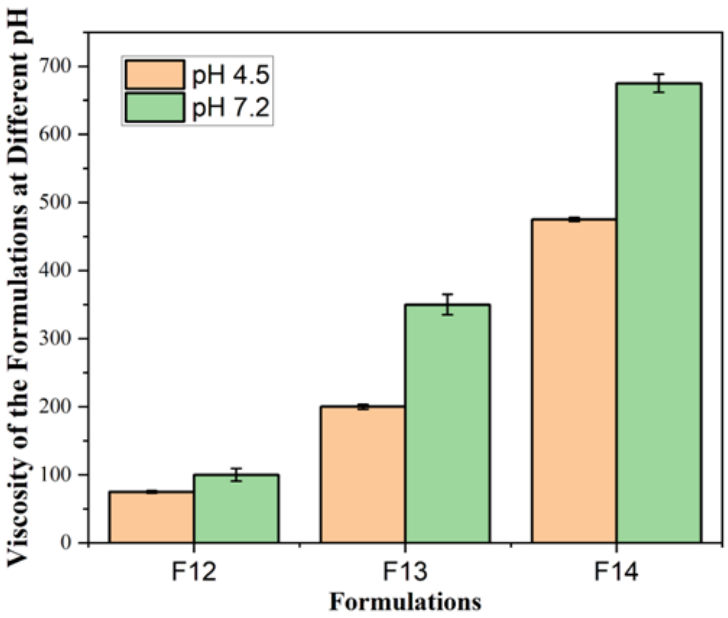

Figure 3. Rheological study (Viscosity study) of *in-situ* gel (n=3)

2.2.5. FTIR analysis

The FTIR spectra of ACZ showed a signal at 3308 cm^{-1} for asymmetric stretching of N–H, stretching bands of N–H (RCONH) at 3184 cm^{-1}, symmetric stretching of N–H (NH_2SO_2) at 3096 cm^{-1}, and stretching of CH_3 and N–H ring at 2916 $cm^{-1}$and 2792 cm^{-1}, respectively. The stretching of C=O or scissoring of NH_2 with primary amide is thought to be responsible for the band at 1680 cm^{-1}. The 1574 cm^{-1} absorbance could be linked to N–H deformation in-plane and 1548 cm^{-1} due to asymmetric stretching of the C=N ring. Stretching of the C=N ring and asymmetric bending of CH_3 were also assigned to the following intensive band, which was found at 1433 cm^{-1}. The remaining bands can be attributed to asymmetric stretching of SO_2 and C–N–C, respectively, at 1364 cm^{-1} and 1316 cm^{-1}. The stretching of the C–N ring is responsible for the peak at 1284 cm^{-1}, while symmetric stretching of SO_2 is responsible for the peak at 1176 cm^{-1}. The FTIR spectra of carbopol 934 revealed a large peak in the range of 3260-2914 cm^{-1}, which corresponded to OH stretching vibration, i.e., O-H and intramolecular hydrogen bonding. Carbonyl C = O stretching band was attributed to the major peak at 1694 cm^{-1}, while –CO stretching vibration was assigned to the peaks at 1459 and 1411 cm^{-1}. A stretching vibration of the C-O-C group was suggested by the band at 1236 cm^{-1}. Out of plane bending of C = CH (bending vibration of aromatic enes) was suggested by the band between 850 and 800 cm^{-1}. The signal at 2890 cm^{-1} and 2821 cm^{-1} were responsible for the stretching vibration band of the OH group and the –CH vibration bands, respectively. A broad absorption band in the range of 3644 – 2918 cm^{-1} was observed in the spectra of sodium alginate. Asymmetric and symmetric stretching vibrations of the COO– groups were attributed to the bands detected at 1584 cm^{-1} and 1405 cm^{-1}, respectively. The signal at 1018 cm^{-1} could be the C–C stretching vibration in sodium alginate. The existence of guluronic and mannuronic acids was further suggested by the stretching vibration bands found at 941 cm^{-1}, 884 cm^{-1}, and 808 cm^{-1}. The FTIR spectra of the physical mixture of ACZ, Carbopol 934, and sodium alginate did not show any significant deviation compared with the individual spectra of the ingredients (Figure 4).

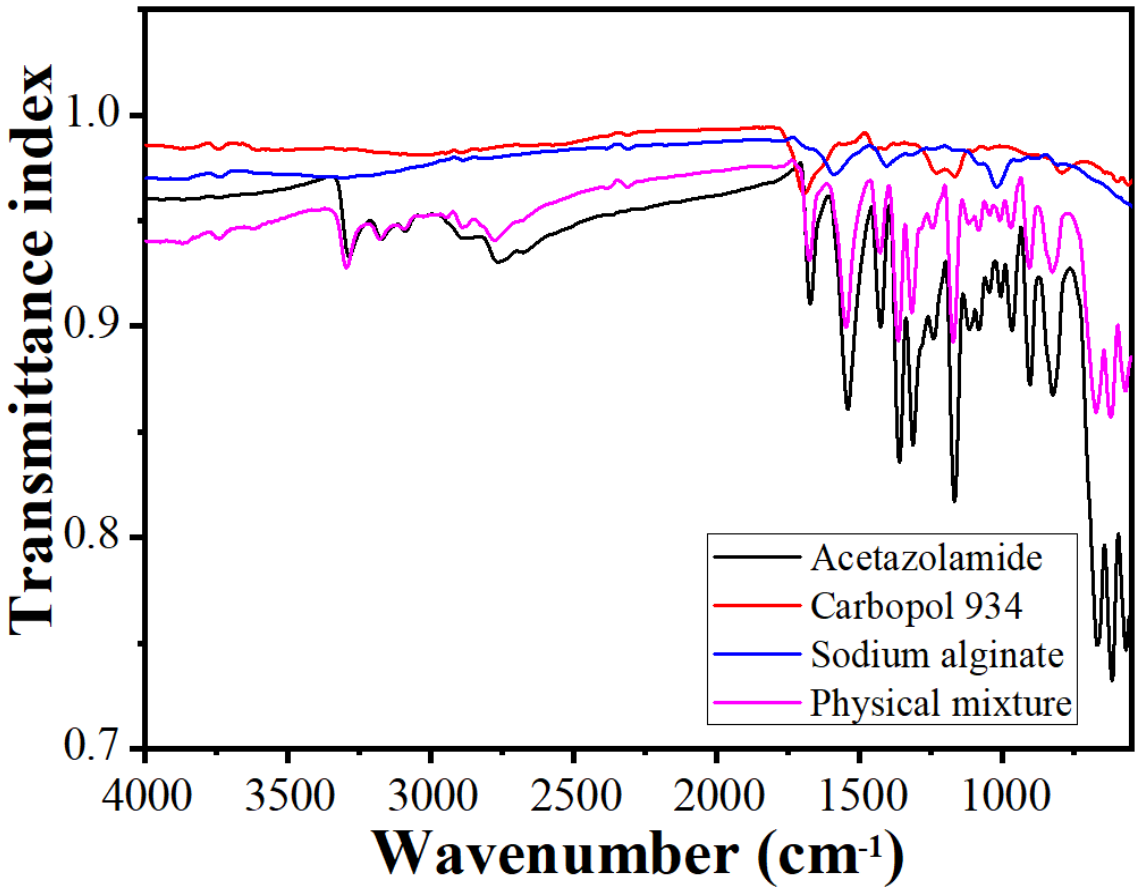

Figure 4. FTIR of the formulations

2.2.6. Sterility testing

Sterility of the ocular formulations is one of the important parameters. The sterility study of all the optimized formulations was conducted. No turbidity was found in all test samples after the specified incubation period (Table 5, Figure 5).

2.2.7. Ocular irritation study

The eye irritation test was evaluated in rabbit model(Draize eye irritation test).The purpose of this test is to determine the ocular tolerance of the formulation [14]. F14 was selected for the eye irritation study. The normal saline (0.1 ml) was instilled into the left eye of the rabbit as control and the test formulation was instilled into the right eye. The result of the rabbit eye irritation study (Figure 6) suggested the good tolerability of the *in-situ* gel as there was no sign of increased tears, redness, chemosis, etc.

Table 5. Results obtained from sterility testing

Formulations	Days of incubation													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
F 12	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
F 13	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
F 14	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

-ve means no changes in the formulations
+ve means changes in formulations (i.e. turbidity, opacity)

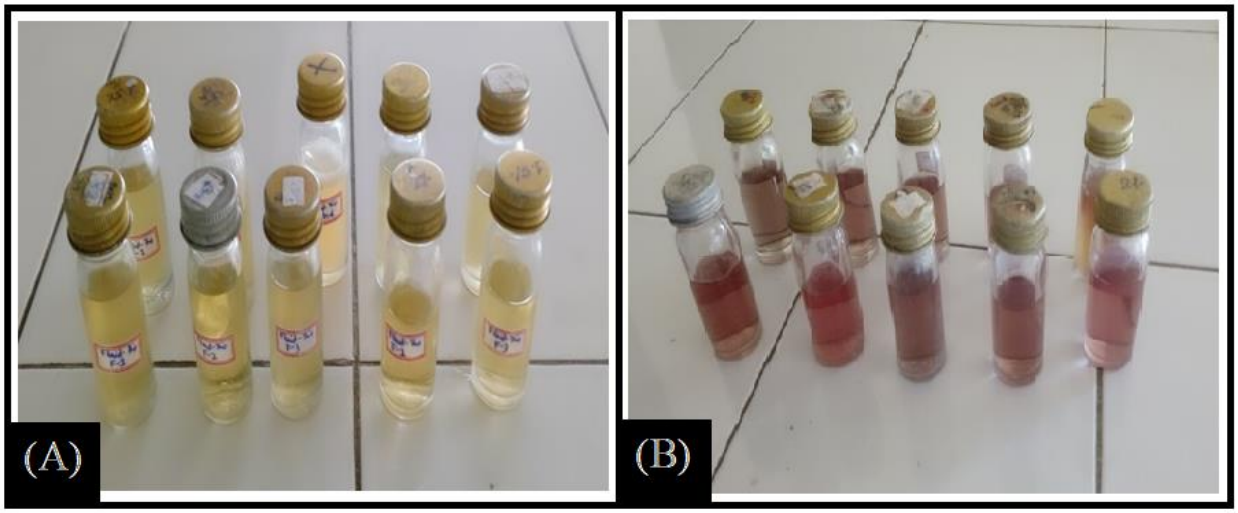

Figure 5. (A) Sterility test of anaerobic and aerobic bacteria, (B) sterility test of fungus.

2.2.8. Ex-vivo corneal permeation study

An ex vivo permeation study was done in Franz's diffusion cell to assess the ocular availability of the drug. The diffusion cell and the Franz diffusion cell have been reported to be efficient methods for the quantification of drug release from the formulations [15]. It was observed that the cumulative percentage drug permeation (CPDP) of F12 in 3 h (1.79±0.050%) was highest and the CPDP was decreased in F13 (1.56±0.027%) followed by F14 (1.19±0.058%) (Figure 7a). The reduction of CPDP could be due to the increased viscosity of the formulation that retards the diffusion of the drug from the formulations. The mechanism of drug release from the polymeric gel was evaluated by fitting the CPDP profile to Korsmeyer-Peppas (KP) mathematical model (Eq. 1; Figure 7b). The permeation exponent (n) seemed to be within the range of 0.45 and 0.89, which indicated to non-Fickian (anomalous) diffusion [16-17]. The release rate constant (K) was observed highest in F13 (0.092±0.004) followed by F12 (0.034±0.003) and F14 (0.029±0.009). The permeation profile was in best accord with the model parameter as indicated by the high correlation value (R^2 >0.999). Again, the permeation profiles were fitted to Peppas-Shalin (PS) mathematical model (Eq.2; Figure 7c) to investigate the drug release mechanism [18-19]. The model parameters were tabulated (Table 6). The constant K_d explains the fickian diffusion, whereas K_r explains the polymer relaxation process.

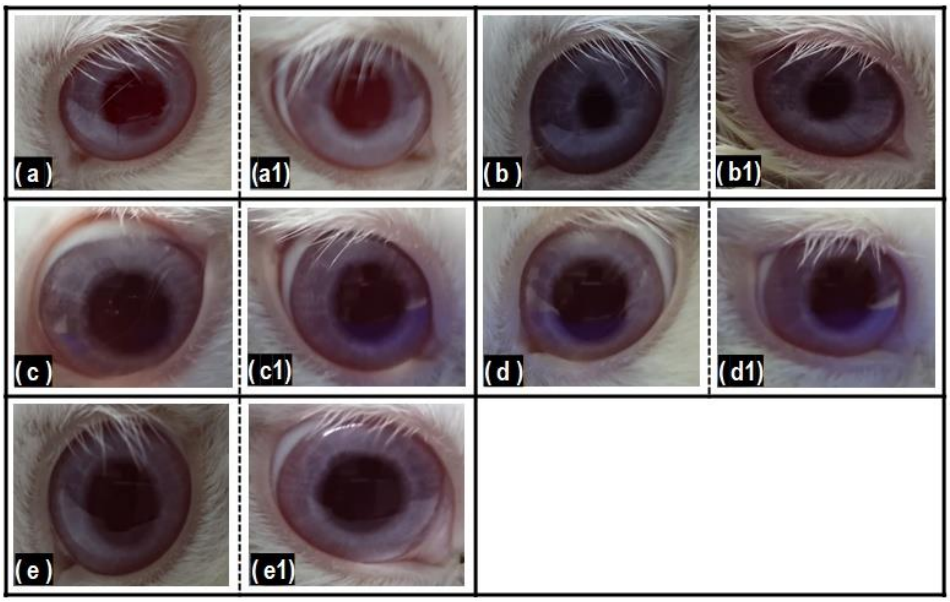

Figure 6. (a) Left eye administered with normal saline at 0 h, (a1) Right eye administered with F13 at 0 h, (b) Left eye administered with normal saline at 1 h, (b1) Right eye administered with F13 at 1 h, (c) Left eye administered with normal saline at 24 h, (c1) Right eye administered with F13 at 24 h, (d) Left eye administered with normal saline at 48 h, (d1) Right eye administered with F13 at 48 h, (e) Left eye administered with normal saline at 72 h, (e1) Right eye administered with F13 at 72 h.

The results of the PS model parameter indicated that the release of the drug from all formulations was due to polymer relaxation only.

$$F = \left(\frac{M_t}{M}\right) = k.t^n \qquad (1)$$

Where$\left(\frac{M_t}{M}\right)$ is the fraction of drug in the receptor compartment at the time "t", K is the release rate constant, and "n" is the diffusion exponent (Table 5).

$$\frac{M_t}{M_0} = K_d.t^m + K_r.t^{2m} \qquad (2)$$

Where, $\left(\frac{M_t}{M_0}\right)$ is the fraction of drug in the receptor compartment at the time "t", "k_d" is the diffusion due to Fickian release, "k_r" is the diffusion due to polymer relaxation, and "m" is the diffusion exponent (Table 6).

2.2.9. Accelerated stability study

An accelerated stability study of F12, F13, and F14 was carried out and the results were presented in table (Table 7). The results confirm that the formulations were stable. There were no significant changes in clarity, pH, and drug content of the formulations during the six months.

3. CONCLUSION

In this study, pH-triggered *in-situ* gel formulations of acetazolamide were successively developed with carbopol 934 and sodium alginate. Among 20 formulations, 3 formulations were selected as per the gelling capacity of the solution. Significant changes in viscosity of the solution system were observed with the changes in pH of the formulation. This suggested the formulations were pH-responsive sol-to-gel system. F13 showed highest increase in viscosity (1.75 times) at pH 7.2 as compared to F14 (1.42 times) and F12 (1.33 times). The gelling time was decreased with the increase of the polymer concentration. The dissolution time of all formulation was nearly equal. The short gelling time and the long dissolution time of the formulations suggested the ideal properties of sol-to-gel system. FTIR study suggested the good compatibility of drug and polymer. The CPDP was highest in F12, followed by F13 and F14. The CPDP was found to be dependent on the viscosity of the formulation. The higher viscosity of the formulation could

slower the diffusion of drug within the formulation. However, the CPDP of F13 was higher up to initial 60 min of the study than F12 and F14, thereafter F13 showed a controlled release. The permeation rate (k) was highest in F13. The KP mathematical model suggested that the drug permeation (n value) was following the non-fickian diffusion. The PS mathematical model suggested the permeation of the drug was due to polymer relaxation process. The in vivo rabbit eye irritation study confirms the well tolerability of the formulation to the ocular tissue. The results of the accelerated stability study showed no significant change in the clarity, pH, and drug content of *in situ* gel. The sterilizing process is validated by the sterility test.

4. MATERIALS AND METHODS

4.1. Materials

ACZ was obtained as a gift sample from Nakoda Chemicals Pvt. Ltd. Sodium Chloride, Sodium bicarbonate, Potassium Chloride, Calcium Chloride, Carbopol-934, and Carbopol-940 (Loba cheme), Fluid thioglycollate medium, Soyabean casein digest medium, HPMC-K4M, and Sodium Alginate (Himedia Laboratories), Benzalkonium Chloride (Merk Specialities Pvt. Ltd.) were purchased from local suppliers.

4.2. Preparation of STF

6.8g of Sodium Chloride, 0.21g of Sodium Bi-carbonate, 1.4g of Potassium Chloride, and 0.08g of Calcium Chloride was weighed properly and shaken till mixed well. After that, the volume was made up to 1000 ml. The pH of STF was between 7.2-7.4.

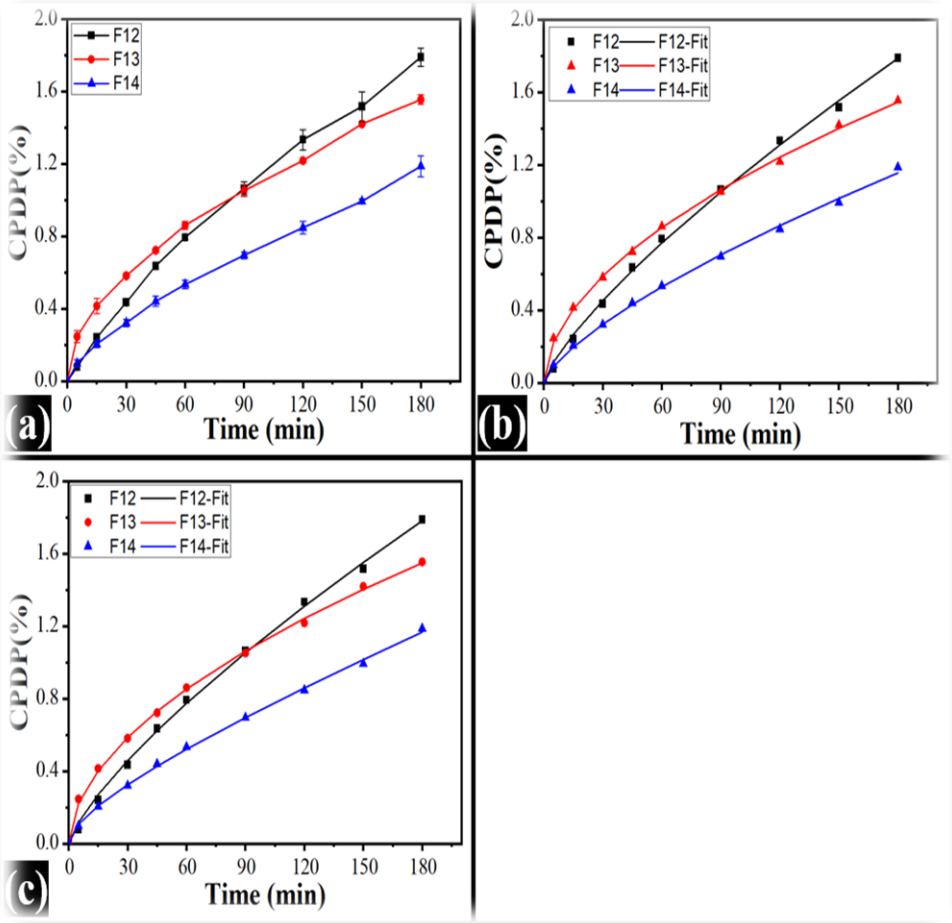

Figure 7. Ex vivo corneal permeation profiles of the formulations: (a) Permeation profile, (b) Korsmeyer-Peppas model fitting, (c) Peppas-Sahlin model fitting.

Table 6. Parameter of drug release

Study	Model	Formulations			
		Parameter	F12	F13	F14
Permeation	Korsemeyer-Peppas	K	0.034±0.003	0.092±0.004	0.029±0.009
		N	0.765±0.026	0.543±0.011	0.713±0.064
		R^2	0.999±0.000	0.999±0.001	0.999±0.001
	Peppas-Shalin	Kd	0.000±0.000	0.069±0.060	0.030±0.007
		Kr	0.034±0.003	0.042±0.042	0.009±0.014
		M	0.382±0.013	0.341±0.062	0.523±0.153
		kd/kr	0.000±0.000	4.611±1.028	35.770±39.167
		R^2	0.999±0.000	0.999±0.000	0.999±0.000

Table 7. Accelerated stability observation

Formulation/parameter	Time (days)	F12	F13	F14
pH	0	4.90±0.02	4.51±0.03	4.30±0.1
	45	4.86±0.11	4.48±0.16	4.29±0.08
	90	4.84±0.05	4.44±0.14	4.26±0.07
	180	4.81±0.06	4.38±0.09	4.25±0.05
Drug Content	0	99.68±0.03	99.18±0.04	99.45±0.02
	45	99.38±0.05	99.16±0.03	99.41±0.02
	90	99.32±0.02	99.05±0.04	99.29±0.03
	180	99.12±0.03	99.01±0.02	99.09±0.04
Visualization through naked eye	0	Transparent	Translucent	Translucent
	45	Transparent	Translucent	Translucent
	90	Transparent	Translucent	Translucent
	180	Transparent	Translucent	Translucent

4.3. Preparation of media

29.75g of fluid thioglycolate media and 30g of soyabean casein digest media was weighed properly and volume was made up to 1000 ml. The media was sterilized by autoclaving at 121 ^{o}C temperature, 15 lbs pressure for 15 min.

4.4. Preparation of formulation

An aqueous solution of different concentrations of carbopol (934 & 940) and HPMC-K4M and sodium alginate (Used as Viscosity enhancer) having formulation code (F1, F2, F3…. F19, F20) were prepared by dissolving in water with excipient (Table 1) at room temperature. An overhead stirrer was used to mix the solution. The gelling capacity of several combinations was evaluated to find the best composition of the sol-to-gel system. Benzalkonium Chloride was added to the optimized formulation as a preservative. The drug was added to the polymer solution with constant stirring. Then, the formulation was transferred to amber color bottle and stored in the refrigerator.

4.4.1. Drug-excipient interaction study

The compatibility study was done by preparing physical mixture of drug and excipient in a ratio of 1:1. The drug was mixed with Carbopol 934 and sodium alginate individually and also with both the excipients. All the powder mix were observed for any physical interaction by visual inspection.

Table 1. Formulation design of the *in-situ* gel

Formulations	Carbopol-934 (%)	Carbopol-940 (%)	HPMC-K4M (%)	Sodium alginate (%)
F1	0.1		0.2	
F2	0.2		0.5	
F3	0.3		0.8	
F4	0.4		1	
F5	0.5		1.5	
F6		0.1	0.2	
F7		0.2	0.5	
F8		0.3	0.8	
F9		0.4	1	
F10		0.5	1.5	
F11	0.1			0.2
F12	0.2			0.5
F13	0.3			0.8
F14	0.4			1
F15	0.5			1.5
F16		0.1		0.2
F17		0.2		0.5
F18		0.3		0.8
F19		0.4		1
F20		0.5		1.5

4.5. Characterization

4.5.1. FTIR analysis

The FTIR of *in-situ* gel was observed by using Alpha-E ATR-FTIR, Bruker, Germany. The samples were analyzed in the range of 4000 to 500 cm^{-1} in the attenuated total reflectance (ATR) mode.

4.5.2. Gelling capacity

One drop of the formulation was put on a watch glass containing 2ml of STF, equilibrated at 37 ± 2 ^{o}C. The time taken to form sol-to-gel, and the dissolution time of the formed gel was recorded.

4.5.3. Appearance and clarity

For the existence of any particle matter, the visual appearance and clarity were evaluated under fluorescent light on a white and black backdrop.

4.5.4. pH determination

The pH of the produced gel formulations was measured using a digital ATC pH meter (L1617 Elico Instrument Mumbai, India).

4.5.5. Rheological study

The viscosity of the formulations was measured by using a Brookfield viscometer. The study was done using spindle No 62, angular velocity ranges from 10-100 rotations per minute (rpm) at room temperature.

4.5.6. Measurement of gelation temperature

In a low-temperature water bath, a transparent beaker containing 20 ml of the formulation and a magnetic bead was placed. The formulation was heated at a steady pace of 2 °C per minute while being constantly stirred. Gelation Temperature (GT) was measured when the magnetic bead stopped moving due to gelation.

4.5.7. Sterility testing

Fluid thioglycollate media and soyabean casein digest media was used for the sterility test of the formulations as per the method described in Indian pharmacopoeia (IP 2007). An anaerobic bacterium (*Escherichia coli*), aerobic bacteria (*Pseudomonas aeruginosa*), and a fungus (*Candida albicans*) were used for the testing. Before the experiment, 100 µL of the organism was poured into the culture bottle and incubated at 32 ± 1 °C for 14 days to examine the microbial growth. The turbidity of the test samples were compared with the control.

4.5.8. Ocular irritancy

Rabbit eye irritation study was done to investigate the corneal tolerance of the *in-situ* gel formulations. For this experiment, 15 nos. of albino rabbits weighing between 1.4 kg to 2.1 kg were used. 100µl of normal saline was administered in the cul-de-sac of the left eye of a rabbit as control and 100µl prepared in situ gel was instilled to the right eye of the rabbit. The rabbit's eyes were then observed for any indications of ocular chemosis at zero hours, one hour, 24 hours, 48 hours, and 72 hours, respectively.

4.5.9. Ex-vivo corneal permeation

Modified Franz diffusion cells were used to evaluate the transcorneal permeation of ACZ. Freshly excised goat eye balls were collected from the local butcher (Salipur, Cuttack, Odisha, India). The eye balls were collected in normal saline (4 °C) and the cornea was separated within 30 min of sacrifice of the goat. The cornea was washed properly with normal saline. The receptor compartment was filled with 12 ml of STF. The cornea was placed in the diffusion cell between the donor and receptor compartments with the epithelial surface facing the donor compartment. 1 ml of the test formulation was placed in the donor compartment and sealed with a cover slip. The receptor compartment was kept at 37±1°C with continuous stirring (300 rpm). The permeation experiment was extended for 180 minutes. The sample was taken at a certain interval of time and analyzed for ACZ using UV spectrometer at λ max 267 nm. The permeation (percent), was calculated as follows:

$$\%\ Permeation = \frac{Amountofthedruginreceptor}{Totalamountofdruginthedoner} X\ 100 \qquad (3)$$

4.5.10. Accelerated stability study

2 ml USP type-I glass ampoules were cleaned with distilled water and dried in the hot air oven. The test samples were filled into the dried ampules, sealed and stored at 40 ± 2 °C, RH 75 ± 5% for six months. At an interval of 0 days, 6 weeks, 3 months, and 6 months, the sample was examined for visual appearance, pH, and drug content.

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