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In situ gelling dorzolamide loaded chitosan nanoparticles for the treatment of glaucoma

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ABSTRACT

The most important risk associated with glaucoma is the onset and progression of intraocular pressure. The objective of this study was to formulate *in situ* gel of chitosan nanoparticles to enhance the bioavailability and efficacy of dorzolamide in the glaucoma treatment. Optimized nanoparticles were spherical in shape (particle size: 164 nm) with a loading efficiency of 98.1%. The *ex vivo* release of the optimized *in situ* gel nanoparticle formulation showed a sustained drug release as compared to marketed formulation. The gamma scintigraphic study of prepared *in situ* nanoparticle gel showed good corneal retention compared to marketed formulation. HET-CAM assay of the prepared formulation scored 0.33 in 5 min which indicates the non-irritant property of the formulation. Thus *in situ* gel of dorzolamide hydrochloride loaded nanoparticles offers a more intensive treatment of glaucoma and a better patient compliance as it requires fewer applications per day compared to conventional eye drops.

1. Introduction

Glaucoma is an age related optic neuropathy characterized by the loss of retinal ganglion cells (RGE), excavation of the optic disc, and progressive visual field loss (Quigley & Broman, 2006; Thylefors & Negrel, 1994). The most important risk factor for the onset and progression of the disease is intraocular pressure (IOP). Although all pharmacological and surgical agents act by reducing intraocular pressure, but in some glaucoma patients, vision loss occurs rapidly due to uncontrollable IOP even after therapeutic IOP control (Thomas, Natalie, & Keith, 2011).

Carbonic anhydrase inhibitors (CAIs) are available as aqueous eye drops solutions (dorzolamide), suspensions (brinzolamide) for topical application and as tablets for systemic drug delivery (acetazolamide and methazolamide) (Mincione, Menabuoni, & Supuran, 2004). The first commercially available topical CAI was aqueous eye drop solution containing 2% (w/v) dorzolamide hydrochloride (Trusopt® from Merck) (Fridriksdottir, Loftsson, & Stefansson, 1997).

There is a high clinical demand for increasing the delivery efficiency of therapeutic drugs in the eye. One of the approaches is *in situ* forming hydrogels which are liquid upon instillation and form viscoelastic gels in response to environmental changes such as pH or temperature (Gratieri et al., 2010; Kalam et al., 2008; Rozier, Maznel, Grove, & Plazonnet, 1989). Nanoparticles such as polyamidoamine (PAMAM) dendrimers, polyguanidilyated dendrimers, and polybutylcyanoacrylate nanoparticles have also been shown to improve ocular drug delivery efficiency (Durairaj, Kadam, Chandler, Hutcherson, & Kompella, 2010; Ibrahim, El-Leithy, & Makky, 2010; Kang, Durairaj, Kompella, O'Brien, & Grossniklaus, 2009; Vandamme & Brobeck, 2005).

The development of novel drug delivery system such as nanoparticles and *in situ* gels has shown a great improvement in ocular bioavailability of the drug. Chitosan, a polyaminosaccharide, shows excellent mucoadhesion that results in improved bioactivity (Busilacchi, Gigante, Mattioli-Belmonte, Manzotti, & Muzzarelli, 2013; Muzzarelli & Muzzarelli, 2005; Rehman, Tavelin, & Grobner, 2011). Chitosan exhibits a cationic nature, owing to its regularly distributed amino groups, and forms various salts (Muzzarelli et al., 2012). Dorzolamide hydrochloride loaded chitosan nanoparticles were prepared by ionotropic gelation method, showing a sustained drug release *in vitro* (Papadimitriou, Bikiaris, Avgoustkis, Karavas, & Georgarakis, 2008). Chitosan and poly-l-lysine loaded nanoparticles were also compared to non-coated nanoparticles and it was found that the unique nature of chitosan and its biodegradability was responsible for bioavailability improvement by enhancing the intraocular penetration of some specific drugs (Das & Suresh, 2010). Sodium alginate, a salt of alginic acid is an excellent polymer in formulations *in situ* gels for ocular drug delivery. It shows a high ocular tolerance and forms solutions of low viscosity at room temperature

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while forms a strong gel on coming in contact with lachrymal fluid (Cohen, Lobel, Trevgoda, & Peled, 1997).

The objective of our study is to design and develop *in situ* gelling polymeric nanoparticles of Dorzolamide Hydrochloride that resides over corneal surface for prolonged period in order to increase the ocular bioavailability and also results in reduction in the dose of the drug which may reduce the side effects. The polymers used in the study *i.e.* chitosan and sodium alginate helps in the sustained drug delivery.

2. Materials and methods

2.1. Materials

The drug Dorzolamide Hydochloride (DZH) (Assay 99.68%) was purchased from BDR Lifesciences Pvt. Ltd., Vadodara. Chitosan (average molecular weight 50 kDa, with degree of deacetylation >80%) was obtained from Sigma–Aldrich, USA. Sodium Alginate (Molecular weight between 75 and 100 kDa, and mannuronic to guluronic acid ratio of 60:40) was purchased from Central Drug House (CDH), New Delhi. Tripolyphosphate (TPP) was supplied by Aldrich Chemicals. Radioactive nuclide ^{99m}Tc was procured from the regional centre of the Board of radiation and Isotope technology, INMAS (Institute of Nuclear Medicine and Allied Sciences), Delhi, India. All the other reagents were of analytical grade.

2.2. Methods

2.2.1. Preparation of in situ gelling polymeric nanoparticles

DZH loaded chitosan nanoparticles were prepared by ionotropic gelation method (Papadimitriou et al., 2008). The drug loaded nanoparticles were prepared by droping the TPP solution (0.1–0.2%, w/v) in chitosan solution (0.125–0.5%, w/v) under continuous magnetic stirring at 1000 rpm (Remi Equipment Pvt. Ltd., Mumbai, India) for 2 h. The pH of the chitosan solution in 2% (v/v) glacial acetic acid was maintained 4–6. 100 mg of DZH was previously dissolved in chitosan solution. The nanosuspension was ultracentrifuged at 18,000 rpm for 30 min (C24Remi, Cooling Centrifuge, Mumbai, India) and the sedimented nanoparticles were washed (three times) by distilled water using the previously described centrifugation approach and then lyophilized by means of Christ Alpha 1–4 lyophilizator (Christ, Osterode, Germany) using 1% (w/v) mannitol as lyoprotectant.

For preparing *in situ* gel the optimized formulation of the nanoparticles (equivalent to 10 mg of drug) were suspended in sodium alginate solution. The optimization of *in situ* gel was carried out by varying concentrations of sodium alginate between 0.5% and 2% (w/w) at constant benzalkonium chloride (0.075%, w/w).

2.2.2. Physicochemical characterization

2.2.2.1. Particle size analysis. It was done using Zetasizer (Model Nano ZS90, Malvern Instrument, UK). 0.1 ml of sample was diluted (100 times) with Millipore water and a UV spectrum was taken. Absorbance of sample should be between 0.01 and 0.1. Light scattering was monitored at 25 °C at a 90° angle. Average particle size and Polydispersity index (PDI) which measure uniformity of particle size within the formulation were determined.

2.2.2.2. Surface morphology. The particle size and surface morphology of the selected formulations were observed using Transmission Electron Microscopy (TEM) (Tecnai G2 S-Twin, FEI, Netherlands) operating at 120 kV at the scale of 500 nm.

2.2.2.3. Rheological study. The viscosities of the preparations were determined in solution as well as gel state by using Brookfield's viscometer, Spindle C-50-1. Each sample was detected for the time period of 60 s. For measuring the viscosity in gel state 1 ml of the solution was converted into gel by the addition of sufficient quantity of Simulated Tear Fluid (STF) at pH 7.4. The viscosities were determined at room temperature *i.e.* 25 °C and the body temperature *i.e.* 37 °C for both solution and gel phase.

2.2.2.4. Pourability of formulation. All the formulations were filled up in the glass vials and their pourability was observed at room temperature using a dropper.

2.2.2.5. pH and clarity. The clarity of the formulation was evaluated by visual inspection. The pH of the *in situ* gels was evaluated using pH metre.

2.2.3. Drug entrapment efficiency and drug loading capacity

The drug loaded nanoparticles (10 ml) were centrifuged at 14,000 rpm for 45 min at 4 °C. The supernatant was collected and the amount of non-entrapped drug was calculated using UV spectrophotometer by observing the absorbance at 253 nm (Papadimitriou et al., 2008)

$$\text{Entrapment efficiency} = \frac{\text{Total drug} - \text{Free drug}}{\text{Total amount of drug}} \times 100$$

$$\text{Loading capacity} = \frac{\text{Total drug} - \text{Free drug}}{\text{Nanoparticle weight}} \times 100$$

2.2.4. X-ray diffraction (XRD)

X-ray diffraction has been used for the study of molecular structure and polymorphism of polymeric nanoparticles. The appropriate amount of drug and freeze dried nanoparticles was mounted in a sample holder and then X-ray investigation of DZH was performed by wide angle X-ray scattering machine (X pert Pro, PANalytical) operating at 45 kV voltage and 40 mA of current. Samples were rotated at 1° rotation per minute. The X-ray source used was copper anode (Kα line).

2.2.5. DSC of nanoparticles

The DSC of formulation was carried out using Differential Scanning Calorimetry (DSC), Perkin Elmer Pyris 6. Pan containing 5 mg of freeze dried nanoparticles was kept in the instrument and heated from 50 to 350 °C at a heating rate of 10 °C. For inert environment in the instrument nitrogen gas was flushed at the rate of 20 ml/min. DSC of pure drug is carried out for the comparison of the thermal behaviour.

2.3. In vitro release study

The *in vitro* drug release was measured using dialysis bag technique (Nagarwal, Kumar, Dhanawat, & Pandit, 2011). 1 ml of the formulation was added to 2 ml of Simulated Tear Fluid (STF pH 7.4) and the gel thus formed was poured onto the inverted test tube with its mouth covered with dialysis bag. The test tube was then immersed in 50 ml of the dissolution media (*i.e.* STF pH 7.4) such that the it lies in the centre of the beaker with its mouth dipped in the media. The medium in the beaker was stirred at 50 rpm and the temperature was maintained at 37 ± 0.5 °C. 1 ml of the sample was withdrawn with the help of a micropipette at regular intervals and it was replaced with 1 ml of the fresh media (Papadimitriou et al., 2008). The absorbance of the samples were measured after appropiate dilution using UV spectrophotometer at the wavelength of 253 nm.

At the same time the *in vitro* release studies of marketed formulation Dorzox (2% ophthalmic solution, 5 ml, Cipla Ltd.) was performed following the same procedure.

2.4. Ex vivo transcorneal permeation studies

The transcorneal permeation studies were done on freshly excised goat cornea obtained from local slaughter house (Sangam Vihar, Delhi). The cornea was carefully removed from the ocular tissue and was washed several times before use in order to remove any proteinous matter, if any. After its removal, the excised cornea was immediately mounted on the Franz Diffusion Cell (1.2 mm dia., 10 ml volume) between the donor and the receptor compartment such that the endothelial surface faced the receptor compartment and epithelial towards the donor. The receptor compartment was filled with 10 ml of fresh STF (pH 7.4). An aliquot of 1 ml of the formulation was placed on the cornea and the receptor fluid was kept at a temperature of 37 °C. Permeation studies were conducted for 6 h (Wadhwa, Paliwal, Paliwal, & Vyas, 2010) and the samples were withdrawn from the receptor compartment at regular intervals and replaced with the same volume of fresh media. The samples were analysed by UV spectrophotometer at 253 nm (Wadhwa et al., 2010).

2.5. Mucoadhesion study of drug loaded in situ gel

The mucoadhesion property of drug loaded nanoparticles, *in situ* gel and *in situ* gelling nanoparticle was analysed using TAXT Plus Texture Analyser (Stable Micro Systems, Surrey, UK; Reddy, Firoz, Rajalakshmi, & Kumar, 2011). 2–3 drops of the formulation were placed on the freshly excised goat cornea (diameter = 2.5 cm obtained from local slaughter house). The cornea was then placed beneath the loading cell and a force of 5 g was applied by the loading cell for 300 s. After this, the loading cell was pulled back and the force required to detach the particles from the cornea (by double sided tape) was determined as mucoadhesive strength. It was calculated according to following formula:

$$\text{Detachment stress } (\text{dynes/cm}^2) = mg/A$$

where m = weight in grams, g = acceleration due to gravity (980 cm/s^2) and A = area of tissue exposed (cm^2).

2.6. Ocular tolerance test (HET-CAM test)

The ocular tolerability of the developed formulation was analysed by using a modified HET-CAM test (Gupta et al., 2007). The HET-CAM test is a qualitative method of assessing the potential irritancy of chemicals. The potential irritancy of compounds may be detected by observing adverse changes that occur in the chorioallantoic membrane of the egg after exposure to test chemicals (Spielmann, 1997). Briefly, fertilized hen's eggs were obtained from a poultry farm. Eggs weighing between 50 and 60 g were selected and divided into three groups each containing three eggs. These eggs were incubated in a humidified incubator at a temperature of 37 ± 0.5 °C for 3 days. The trays containing eggs were rotated manually in a gentle manner after every 12 h. On the third day, 3 ml of egg albumin was removed by using sterile techniques from the pointed end of the egg. The hole was immediately sealed by 70% alcohol-sterilized Parafilm (American Can Company, Neenah, Wisconsin) with the help of a heated spatula. The eggs were kept in the equatorial position for the development of chorioallantoic membrane (CAM) away from the shell. The eggs were candled on the fifth day of incubation, and every day thereafter nonviable embryos were removed. On the tenth day, a window (2 cm × 2 cm) was made on the equator of the eggs through which formulations (0.5 ml) were instilled directly onto the CAM surface and left in contact for 5 min. The membrane was examined for vascular damage as the sign of haemorrhage, hyperemia and coagulation. Normal saline (0.9% NaCl solution) was used as a control as it is reported to be practically nonirritant. The scores were recorded according to the scoring schemes as shown in Table 1.

Table 1
Scoring chart for HET-CAM test.

Effect	Score	Inference
No visible haemorrhage	0	Nonirritant
Just visible membrane discoloration	1	Mild irritant
Structures are covered partially due to membrane discoloration or haemorrhage	2	Moderately irritant
Structures are covered totally due to membrane discoloration or haemorrhage	3	Severe irritant

2.7. Gamma scintigraphic study

The formulation was labelled with radioactive nuclide ^{99m}Tc. For labelling, 100 μg of stannous chloride was mixed with the formulation (C2S4). To this, ^{99m}Tc (2–3 mci) was added and mixed properly. The labelling efficiency of the formulation was determined using instant thin layer chromatography (ITLC). A drop of the formulation was dropped onto the ITLC strip, which was run in 100% acetone as mobile phase. The strip was then dried and cut into many pieces and the radioactivity was determined using Gamma counter (Capintec, CAPRAC-R, CII, USA). The labelling efficiency of the drug greatly depends upon the amount of reducing agent (stannous chloride) which was already optimized.

In vivo pre corneal retention time of the formulation was assessed using Gamma scintigraphy. Male Albino rabbit of (3–4 kg) was used for the study. Animals were procured from the animal house of INMAS (Delhi, India) and having free access to food and water. The study was carried out with utmost care in order to

Table 2
Optimization of nanoparticles.

Formulation code	Chitosan concentration (%)	TPP concentration (%)	Particle size (nm)	Polydispersity index (PDI)	Entrapment efficiency (%)	Drug loading (%)
A	0.125	0.2 (2 ml)	687.5 ± 17.57	0.647	94.2	15.9
A1	0.125	0.2 (3 ml)	203.1 ± 10.03	0.386	97.2	18.4
B	0.15	0.2 (2 ml)	492.6 ± 13.28	0.478	95	16.5
B1	0.15	0.2 (3 ml)	613 ± 14.89	0.526	96.8	15
C	0.175	0.175 (1 ml)	270.8 ± 19.2	0.578	93.9	17
C1	0.175	0.175 (2 ml)	286 ± 13.69	0.386	94.1	16.89
*C2	0.175	0.175 (4 ml)	164.0 ± 10.2	0.240	98.1	19.7
D	0.5	0.1 (1 ml)	4329.78 ± 0.72	1.2	64.7	8.7
D1	0.5	0.1 (2 ml)	3865.1 ± 0.93	0.98	68	10
E	1	0.2 (1 ml)	785 ± 20.8	0.872	75.3	12
E1	1	0.2 (2 ml)	790 ± 0.16	0.834	66.6	14.8

* Is the optimized formulation selected for further study.

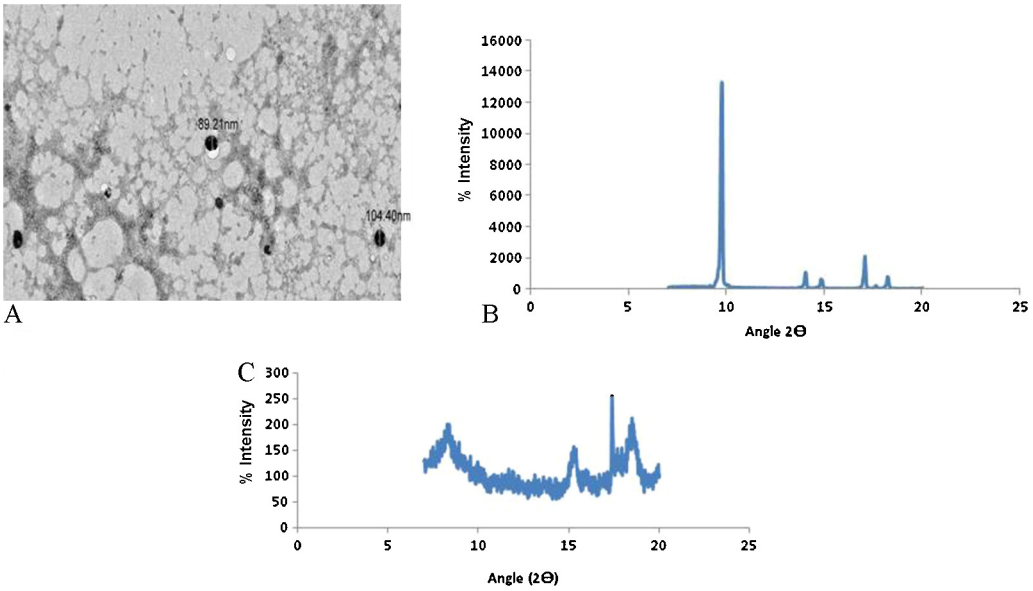

Fig. 1. (A) Particle size and surface morphology of C2, (B) XRD of dorzolamide, and (C) XRD of nanoparticle.

ensure that animals were treated in the most human and ethically acceptable manner.

In order to determine the pre corneal retention time of the radio labelled formulation, rabbit was first anaesthetized using Diazepam given intramuscularly. The rabbit was then tied to a wooden base and was positioned 5 cm in the front of probe with supine position of the Gamma Camera (SIEMENS, Symbia true point, SPECT.CT), auto tuned at 140 keV radiation of ^{99m}Tc. 20 μl of the formulation was instilled into the cul-de-sac region of the left corneal surface of the rabbit's eye. The eye was blinked three times in order to ensure uniform distribution of the formulation. Recording was started after 5 s of the instillation and continued for 30 min using 64 × 64 pixel matrix. Individual 60 frames at every 30 s were captured by dynamic imaging process. A single whole body static image was also taken after 2 h of instillation of the formulation.

3. Results and discussion

3.1. Preparation of in situ gelling polmeric nanoparticles

The optimization of nanoparticles was done by taking into considerations of various parameters such as particle size, drug loading capacity, polydispersity index (PDI). The optimization of various concentrations of chitosan and TPP is shown in Table 2. The various formulation of gel was subjected to rheological studies in order to select the optimum formulation on the basis of the rheological studies at various temperatures and angular velocity. The optimization of *in situ* gel for various parameters is shown in Table 3. Out of seven formulations, at two concentration of sodium alginate (1.25% and 1.5%, w/w) the gel showed appropriate viscosity and pourability so these two formulations *i.e.* S4 and S5 were selected and evaluated for *in vitro* release. The optimized drug loaded nanoparticle was dispersed into optimized *in situ* gel and evaluated for various parameters as shown in Table 4.

3.2. Physicochemical characterization

It was observed that on increasing the chitosan: TPP ratio, the particles of larger sizes were obtained. The particle sizes and PDI of the formulations C2 (62.31% yield) was found to be 164.0 nm with PDI 0.240 which is smallest of all formulations prepared.

The TEM image of the C2 formulation shows the particle size as well as the morphlogy of the nanoparticles in Fig. 1A. The nanoparticles were found to be spherical in shape with particle size of 89.2 nm.

The viscosities of the formulations were found to increase as the concentration of sodium alginate increased (Table 3). The viscosities of the formulations were increased after gelling at 25 and 37 °C. Out of seven formulations, S4 and S5 were found to have appropriate viscosity and pH for ophthalmic use. These formulations (S4 and S5) were selected for *in vitro* release study.

All the *in situ* gel formulations were found to be easily pourable at room temperature. The pH of the *in situ* gel formulations was given in Table 3. The clarity of the formulation was appropriate. The pH of the optimized formulation was 6.7 as shown in Table 3.

3.3. Drug entrapment efficiency and drug loading capacity

The drug entrapment efficiency was also considered as an important parameter in the selection of the chitosan nanoparticles. At a constant chitosan concentration, with increasing concentration of TPP, entrapment efficiency increases. It was found that most of the formulations showed entrapment efficiency of more than 50%. At certain low concentration of chitosan (0.175%, w/w) and TPP (0.175, w/w) the formulation C2 has maximum entrapment efficiency of 98.1% (Table 2). C2 has shown the maximum drug loading capacity of 19.7% amongst all the formulations (Table 2).

3.4. XRD of nanoparticles

X-ray diffraction has been used for the study of molecular structure and polymorphism of polymeric nanoparticles. The XRD pattern of the pure drug as shown in Fig. 1B exhibited sharp peak at 2θ angle 9.89 indicating the crystalline nature of drug. However, no characteristic peaks were observed when the lyophilized polymeric nanoparticles were subjected to XRD as shown in Fig. 1C. The XRD graph of nanoparticles showed many depressed and broader peaks with decreased intensity. This is due to the transformation of the state of drug from crystalline to amorphous form (Papadimitriou et al., 2008).

Table 3
Rheological studies data for optimization of *in situ* gel.

S. No	Formulation code	% w/w of sodium alginate	Angular velocity (rpm)	Viscosity before gelling (cps)		Viscosity after gelling (cps)		pH ($n = 3$)	Selected/rejected
				25 °C	37 °C	25 °C	37 °C		
			10	40	36	62	58		
1.	S1	0.5	20	27	25	55	51	5.8	R
			50	26	23	53	48		
			10	56	50	100	95		
2.	S2	0.75	20	45	40	76	68	6.4	R
			50	39	30	65	60		
			10	63	60	110	106		
3.	S3	1.0	20	55	50	96	90	6.6	R
			50	48	45	89	85		
			10	80	71	250	246		
4.	*S4	1.25	20	69	60	220	205	6.7	S
			50	50	42	200	187		
			10	85	80	300	293		
5.	*S5	1.50	20	79	69	287	273	6.5	S
			50	64	54	275	264		
			10	89	80	372	368		
6.	S6	1.75	20	81	76	360	344	6.4	R
			50	72	65	341	338		
			10	90	82	400	396		
7.	S7	2.0	20	82	73	346	333	6.8	R
			50	70	61	327	313		

* S4 is selected for further study.

3.5. DSC of nanoparticles

DSC was carried out in order to determine the melting point and crystallization behaviour of the pure drug and the polymeric nanoparticles. The DSC of pure DZH in Fig. 2A exhibits a single sharp endothermic peak at 279 °C which showed that the drug was crystalline in nature. While, the DSC curve of DZH loaded nanoparticles in Fig. 2A did not show any endothermic peak. The reason for this might be the conversion of the drug from crystalline to amorphous form after incorporation into the polymer. The amorphous form was thought to have higher energy with increased surface area subsequently resulting in higher solubility, dissolution rate and bioavailability (Agnihotri & Vavia, 2009; Pignatello et al., 2002).

3.6. In vitro release study

The *in vitro* release data of various formulations is shown in Fig. 2B. The *in vitro* release studies of optimized formulation of nanoparticle (C2) and *in situ* gel (S4 and S5) depicts the release of about 74.90% and 62–70% of drug respectively in 8 h. The *in vitro* release from marketed formulation (drug in solution) shows more than 98% release of the drug in same time. *In situ gel* formulations (S4 and S5) shows more sustained release than C2 as the release of drug is controlled by the thick viscous gel in which drug was dispersed.

In situ gel formulation S4 was found to exhibit more sustained drug release characteristics as compared to S5, therefore it was selected for the preparation of *in situ* gel nanoparticle.

The *in vitro* release from optimized formulation C2S4 showed a release of 58% in 8 h thus making it superior over the nanoparticles and *in situ* gel alone.

The formulation also showed a slow and sustained release as it fitted best in the zero order plots.

3.7. Ex vivo transcorneal permeation study

The *ex vivo* transcorneal permeation studies of the optimized formulation and marketed formulation was shown in Fig. 2C. The *ex vivo* permeation of the optimized formulation (C2S4) showed a drug permeation of 35.80% within 2 h which was much lower compared to the marketed formulation with a permeation of 75.30% at same time. This permeation difference between the developed formulation (C2S4) and marketed formulation is attributed due to the slow release of drug from nanoparticle and the release was further controlled by the gelling matrix of the polymer (Calvo, Vila-Jato, & Alonso, 1996).

3.8. Mucoadhesion study

The mucoadhesive force required for the detachment of drug loaded nanoparticle (C2), *in situ* gel (S4) and of the *in situ* gelling polymeric nanoparticles (C2S4) was found to be 20 g, 10 g and

Table 4
Evaluation of *in situ* gelling polymeric nanoparticles.

Formulation code	Angular velocity	Viscosity (cps)				pH ($n = 3$)	Time of gelling (s)	Clarity		Pourability
		25 °C (sol)	25 °C (gel)	37 °C (sol)	37 °C (gel)			Sol	Gel	
	10	82	330	73	319					
C2S4	20	73	318	67	310	6.7	10	Clear, viscous	Clear, strong	Easily pourable
	50	60	304	54	298					

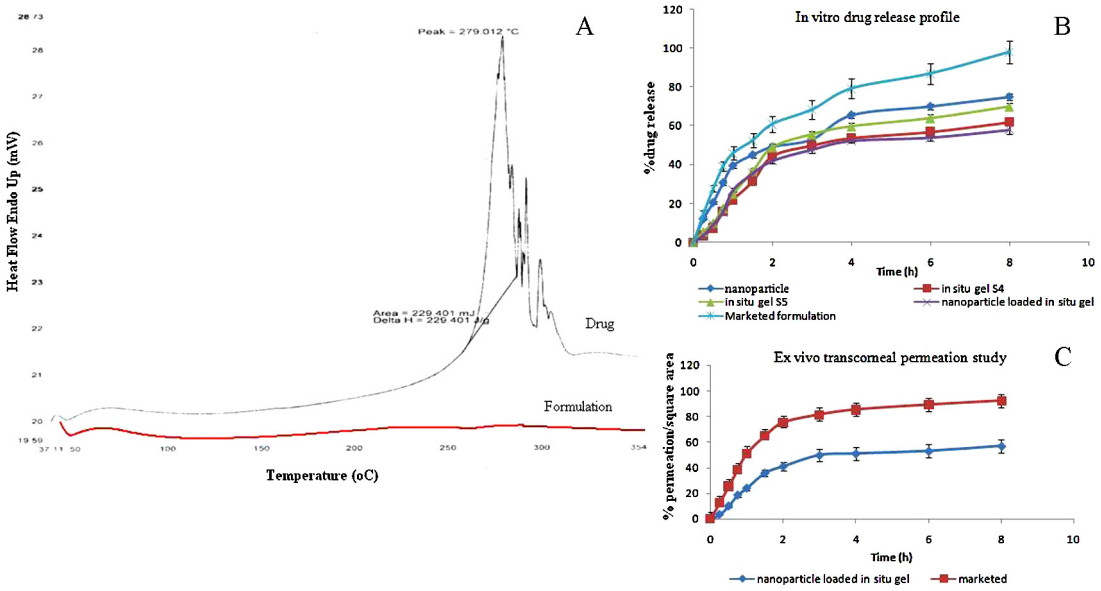

Fig. 2. (A) DSC curves of dorzolamide hydrochloride and dorzolamide loaded freeze dried nanoparticles, (B) *in vitro* release profile of various formulations, and (C) *ex vivo* transcorneal permeability study.

30 g, respectively as shown in Fig. 3A. The mucoadhesive strength was calculated and it was found that the formulation C2S4 was having highest mucoadhesion (11,760 dynes/cm^2) as compared to nanoparticle (7840 dynes/cm^2) and *in situ* gel (3920 dynes/cm^2). The increase in mucoadhesive strength of C2S4 formulation could be due to the cumulative effect of chitosan and alginate.

3.9. Ocular tolerance test (HET-CAM test)

A cumulative score of 0 was obtained for normal saline. The cumulative scores for the formulation C2S4 was found to be 0.33 which is below 1 accounting for practically non-irritant (Table 5). Therefore, the formulation is considered to be safe and non-irritant.

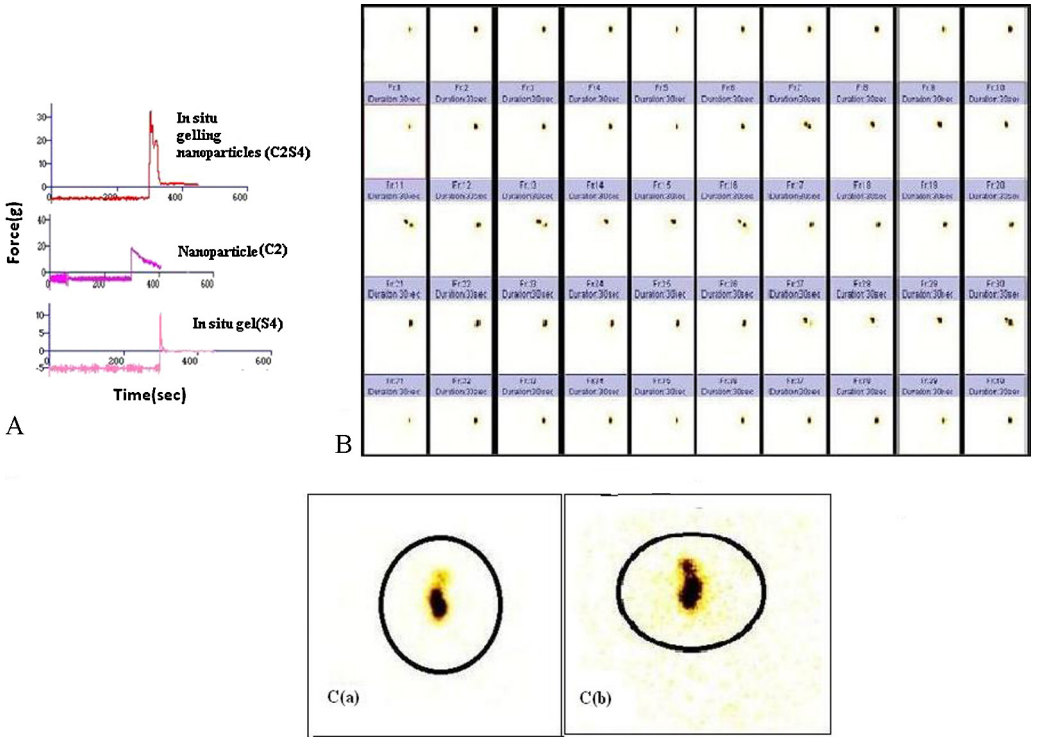

Fig. 3. (A) Graph illustrating the mucoadhesive strength, (B) dynamic images of formulation C2S4 in first 25 min, and (C) static images of C2S4 after 2 h of instillation. (a) Anterior region of right eye, (b) posterior region of right eye.

Table 5
Score obtained in HET-CAM test.

Formulation	Egg	Scores									Cumulative scores for 5 min
		Haemorrhage (A) Time (min)			Hyperaemia (B) Time (min)			Coagulation (C) Time (min)			A + B + C
		0.5	2	5	0.5	2	5	0.5	2	5	
Formulation C2S4	Egg 1	0	0	0	0	0	0	0	0	0	0.33
	Egg 2	0	0	0	0	0	0	0	0	1	
	Egg 3	0	0	0	0	0	0	0	0	0	
	Mean	0	0	0	0	0	0	0	0	0.33	
Normal saline	Egg 1	0	0	0	0	0	0	0	0	0	0
	Egg 2	0	0	0	0	0	0	0	0	0	
	Egg 3	0	0	0	0	0	0	0	0	0	
	Mean	0	0	0	0	0	0	0	0	0	

3.10. Gamma scintigraphic study

The gamma scintgraphy of optimized formulation (C2S4) was carried out by labelling the formulation with ^{99m}Tc. The labelling efficiency when measured by Gamma counter was found to be 954.2 kcpm (kilo counts per minute). The images which were captured using the Gamma camera showed that the formulation was retained very well in the pre corneal region during the first 25 min of instillation which was observed through the dynamic imaging (Fig. 3B). The static image captured after two hours showed that the formulation was retained at the corneal surface with no significant radioactivity in the systemic circulation (kidney & bladder). Fig. 3C, (a) and (b) show a good retention for formulation C2S4 in the anterior and posterior chamber of eye respectively which was due to the mucoadhesive property of polymers (chitosan & alginate) that prolonged residence time on corneal region so intercellular and intracellular permeation has taken place.

4. Conclusion

The optimized formulation C2S4 exhibited good gelling property with 98.1% entrapment efficiency. *In vitro* release study shows the amount of drug release from the formulation with respect to time while *ex vivo* permeation study shows how rapidly the amount drug permeated across a unit area of biological membrane. The *ex vivo* transcorneal permeation studies reveals that the formulation C2S4 showed slower and sustained permeation of drug (35.50%) as compared to fast and rapid (86.34%) permeation for the marketed formulation. Further the mucoadhesion and ocular tolerance tests showed the mucoadhesive and non-irritant nature of the formulation. The Gamma scintigraphy study for the formulation on albino rabbits showed long retention of the formulation into the eye. Thus, the formulation showed a good pre corneal retention property which lead to improvement in the bioavailability of DZH.

On the basis of the evaluation and the comparison with the marketed formulation of DZH, the *in situ* gelling formulation (C2S4) is thus considered better delivery system for the treatment of glaucoma.

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