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Original Article

Formulation and release of timolol maleate from ocusert based on gelatin-N-(3-dimethylaminopropyl)-N-ethylcarbodiimide polymer

[Formulación y liberación de maleato de timolol de ocusert a base de polímero gelatina-N-(3-dimetilaminopropil)-N-etilcarbodiimida]

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Abstract

Context: Glaucoma is a disease of the eyes characterized by an increase in the intraocular pressure. Timolol maleate is commonly used as eye drops for chronic glaucoma treatment.

Aims: To formulate an ocusert (novel ophthalmic drug delivery systems) in order to overcome disadvantages of eye drops such as patient's noncompliance and drainage of the administered solution. Also, to improve treatment outcomes by keeping sustained release of constant amount of timolol maleate and to avoid repeated administration of conventional eye drops.

Methods: Timolol maleate ocuserts were formulated using cross-linked gelatin polymer which was prepared using N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) and N-hydroxysuccinamide (NHS). Different ocusert formulas were prepared (M1-M17) by varying concentrations of EDC, different temperatures and time for the cross linking as per central composite design. Physicochemical characteristics of drug loaded ocuserts were investigated.

Results: Selected formula (M8) exhibited an excellent *in vitro* drug release results which was extensively evaluated. Fourier transform infrared spectral scan indicates no incompatibility exists between the drug and the polymer used. M8 formula was evaluated *in vivo* by assessing the eye irritancy, drug release and therapeutic effect when placed in the *cul-de-sac* of rabbit's eyes and was compared with conventional eye drops therapy.

Conclusions: There was a correlation between *in vivo* and *in vitro* release of timolol maleate which is associated with a decrease of glaucoma induced by dexamethasone eye drops in experimental rabbits. The data established the potential of ocusert to improve the therapeutic delivery of timolol maleate and offers a promising option in glaucoma treatment.

Keywords: animal studies; glaucoma; gelatin; ocusert; N-(3-dimethylaminopropyl)-N-ethylcarbodiimide; N-hydroxysuccinamide; timolol maleate.

Resumen

Contexto: El glaucoma es una enfermedad de los ojos caracterizada por un aumento de la presión intraocular. El maleato de timolol se usa comúnmente como gotas para los ojos para el tratamiento del glaucoma crónico.

Objetivos: Formular un ocusert (nuevos sistemas de administración de fármacos oftálmicos) para superar las desventajas de las gotas para los ojos, como el incumplimiento del paciente y el drenaje de la solución administrada. Además, para mejorar los resultados del tratamiento manteniendo la liberación sostenida de una cantidad constante de maleato de timolol y para evitar la administración repetida de gotas oftálmicas convencionales.

Métodos: Se formularon ocusertes de maleato de timolol usando polímero de gelatina reticulado que se preparó usando N- (3-dimetilaminopropil) -N-etilcarbodiimida (EDC) y N-hidroxisuccinamida (NHS). Se prepararon diferentes fórmulas de ocusert (M1-M17) variando las concentraciones de EDC, diferentes temperaturas y tiempo para la reticulación según el diseño del compuesto central. Se investigaron las características fisicoquímicas de los ocusertes cargados de fármaco.

Resultados: La fórmula seleccionada (M8) mostró excelentes resultados de liberación de fármaco *in vitro* que fueron evaluados exhaustivamente. La exploración espectral infrarroja por transformada de Fourier indica que no existe incompatibilidad entre el fármaco y el polímero utilizado. La fórmula de M8 se evaluó *in vivo* evaluando la irritación ocular, la liberación del fármaco y el efecto terapéutico cuando se colocó en el fondo de saco de los ojos del conejo y se comparó con la terapia convencional con gotas oftálmicas.

Conclusiones: Hubo una correlación entre la liberación *in vivo* e *in vitro* de maleato de timolol que se asocia con una disminución del glaucoma inducido por el colirio de dexametasona en conejos experimentales. Los datos establecieron el potencial de ocusert para mejorar la administración terapéutica de maleato de timolol y ofrece una opción prometedora en el tratamiento del glaucoma.

Palabras Clave: estudios en animales; glaucoma; gelatina; ocusert; N- (3-dimetilaminopropil) -N-etilcarbodiimida; N-hidroxisuccinamida; timolol maleato.

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INTRODUCTION

Glaucoma is characterized by an increase in the intraocular pressure (IOP), which may lead to blindness if it is not treated. It can be treated by surgical procedures or treatment with different drugs. Aqueous humor is an aquatic fluid in the eye's front and back chambers. Liquid deposition results in an increase in the event of a channel being blocked. Abnormal aqueous humor drainage can lead to higher IOP. This can lead to glaucoma development and may lead to loss of vision. IOP increase of aqueous humor can be treated either by increasing drainage or by decreasing secretion by certain medications such as β-blocker. Glaucoma leads to deterioration of the optic nerve caused by elevated fluid pressure of the eye. There are no early symptoms or pain in most people with glaucoma. In order to keep a constant amount of anti-glaucoma medication, repeated administration of the standard eye drops is essential (Weinreb et al., 2014).

Glaucoma is generally described as either a closure-angle or open-angle glaucoma. For open-angle glaucoma, a spatial blockage develops within the trabecular meshwork, which prevents the drainage of aqueous humor. Aqueous drainage disability increases IOP to a value of 25-35 mm Hg (normal IOP is 10 to 20 mm Hg), which usually suggests partial blocking. In closure-angle glaucoma, pupillary blockage of aqueous humor outflow causes excessive IOP and is more serious. A pupillary block, a narrowed anterior chamber angle and a convex iris are the fundamental conditions that lead to an acute angle closure attack. Due to the continuous secretion of aqueous humor, pressure in the posterior chamber forces the iris to bulge forward. This can progress towards completing the blockage of drainage (Imam et al., 2009).

Timolol maleate was well tolerated by most patients, with fewer subjective complaints compared to comparative medicines and some patients were treated without serious adverse effects or loss of effectiveness for long periods. Timolol maleate has a significant advance in the therapy of glaucoma. The maximum effect of timolol maleate on IOP was recorded in previous studies using a concentration of 0.5% eye drops. The mechanism for reducing IOP by topical timolol maleate is mainly due to a decrease in aqueous humor production. In single-dose study, peak plasma and aqueous humor concentrations were 0.188 µg/mL and 2.47 µg/mL, respectively, which were achieved within 30 minutes after an ocular administration of a 0.5% solution (Heel et al., 1979).

The ophthalmic insert is defined as a solid or semisolid sterile preparation with suitable size and shape of the purpose for which intended. It stays in the *cul-de-sacs* and ensures continuous release throughout the desired treatment period. The release and permeability of the drug are important factors for its bioavailability. The drug permeability must be improved to increase the therapeutic efficiency and to maintain the effects over a desired period of time. Novel drug delivery systems have the ability to release drug at a slow steady rate during the required interval (Preeti et al., 2013).

Due to gelatin outstanding biocompatibility, ease of processing and low-price accessibility, it has been used in pharmaceutical manufacturing, cell culture and engineering of tissues. Gelatin has been widely assessed over the past decade for a broad spectrum of eye applications that serve as cell-sheet carriers, bio-adhesives and bio-artificial grafts (Rose et al., 2014). The gelatin-based formulation such as ocusert can solve certain issues arising from standard ophthalmic dosage forms such as eye drops including frequent administration, incorrect dose and problem related to patient compliance.

The new technique for the formulation of ophthalmic antiglaucoma drugs uses inert, safe excipients and lower cost materials than those available in the marketed corresponding devices (Gaudana et al., 2009). Chemical cross-linkers influences ocular biocompatibility can facilitate the development of suitable cross-linked gelatin substances to enhance cell and drug therapy. Chemical cross-linked hydrogels show longer durability, greater

stability and higher mechanical characteristics (tensile, shear, bending, etc.) especially in comparison to physically crosslinking hydrogels (Lai, 2010). By inserting functional monomers into the polymer system or by linking the two polymer chains with a crosslinker, chemical crosslinked hydrogels are produced. Chemically crosslinking reaction processes are based on crosslinking of functional groups of polymers (-OH, -COOH, or NH_2, etc.) with crosslinkers such as N-(3 dimethylaminopropyl)-N-ethyl carbodiimide (EDC) and N-hydroxysuccinamide (NHS) or aldehydes (such as glutaraldehyde or adipic acid dihydrazide). Such crosslinkers are thought to be a zero-length crosslinker that induces carboxylic acid groups and afterward creates amide bonds with amino groups of gelatins. The addition of NHS to the EDC reaction mixture conducted to avoid the possible side reactions and to improve crosslinking efficacy. EDC/NHS are zero-length cross linker used for the preparation and modification of gelatins. This modifying process is known to become less toxic since it just cross-links biopolymers without introducing external materials into biopolymers (Kuijpers et al., 2000). EDC-Gel disks demonstrate superior biocompatibility and are well accepted without inducing irritation or harmful effects (Lai and Li, 2010). The degree of swelling of these structures ranged from 10% to 95%, based on their composition, hydrophilicity, and degree of crosslinking. These devices, despite their high-water content, can be used to release hydrophilic and hydrophobic drugs (Farris et al., 2010). The crosslinked networks can be swelling up to 90% by water, and their thermodynamic, swelling, mechanical and diffusion nature are well known (Yasuda and Lamaze, 1971).

Recent trends are in vogue as follows: Ocular inserts, polymer-based delivery plans, collagen shields, muco-adhesive dosage forms and drug pre-soaked hydrogel type contact lens. Definitive knowledge of the complexities of tissues, physiological and multi-compartmental pharmacokinetics in normal and pathological circumstances, would have significantly enhanced further progress in this field (Kumar et al., 2013). The present investigation is focused to develop ocuserts using central composite design and evaluate for their characteristics, *in vitro* and *in vivo*.

The aim of this work was to prepare ocuserts using chemically cross-linked gelatin and evaluate the release studies of the drugs. The ocuserts would increase contact time, which led to prolong the release of the drug, decrease systemic and local side effects, and decrease frequent administration, providing appropriate and accurate dosing. *In vitro* release studies and *in vivo* evaluation of timolol maleate ocuserts for their effect on IOP of rabbit's eyes were studied.

MATERIAL AND METHODS

Materials

Timolol maleate (99.5%) was obtained as a gift from Dar Al Dawa Pharmaceutical Company, Amman, Jordan. Type B gelatin raw material (100-115 millimoles of free carboxyl groups per 100 g of protein), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, purity ≥98.0%), N-hydroxysuccinamide and phosphate buffered saline tablets were purchased from Sigma-Aldrich, USA.

Animal studies

The use of animals in the project had been approved by Faculty and University Graduate Research Committees, letter numbers 2019-2018/7 dated 17/03/2019 and decision number 274, dated 21/03/2019. Animals (male New Zealand rabbits) were handled humanely to minimize pain and discomfort as per guideline. The animals were acclimatized to the typical laboratory conditions in cross ventilated animal house, fed with standard pallet diet and water at a temperature of 25 ± 2°C, relative humidity of 44 to 56% and light and dark cycles of 12:12 h. Appropriate measures were taken during the experiment and localized inflammatory symptoms in eyes were recorded. None of the animals were sacrificed in this study.

Preparation of the ocuserts

Gelatin hydrogel disks were prepared using solvent casting techniques as described elsewhere

(Lai et al., 2006). Briefly, in order to achieve hydrogel sheets (about 0.3 mm in thickness), an aqueous sterile solution of 10% gelatin (w/v) was casted into sterilized glass Petri-dish and air-dried for 3 days at 25ºC. Using a 6-mm diameter sterile biopsy punch the gelatin hydrogels have been cut out to obtain the oval shaped hydrogels. The resulting gelatin hydrogels were cross-linked by directly immersing the samples in different concentrations of EDC/NHS solution (Lai, 2013). The cross-linking reaction was allowed to proceed at different temperatures for various periods. After the reaction, the resulting hydrogel sheets were washed thoroughly with sterile double-distilled water to remove excess EDC/NHS, then dried in air at room temperature for 24 h. The drug loading is achieved by directly immersing the resulting samples into sterile solution of timolol maleate (4%) for 24 h at room temperature.

Design of formulation

Central composite design (CCD) was chosen to optimize the formula and understand the effect of various parameters such as the concentration of EDC, immersion time in EDC and temperature on integrity, quality and percent drug loaded. The CCD consists of 17 experiments, which include eight replicates of factorial points, three replicates of center points and six axial points at extreme levels. The parameters are shown in Table 1. The data were analyzed using Design Expert 8.0.6 (Statease, USA) and MODDE 12.1 (Sartorius Inc., USA) software.

Wavelength (λ) for the chromatography

From the UV spectrum recorded on UV-visible spectrophotometer (Shimadzu UV-1800), 300 nm was selected as the wavelength suitable for the analysis of timolol maleate. Timolol maleate 50 µg/mL solution in methanol (50%) was injected into the HPLC system and the peak parameters were monitored at 300 nm.

Chromatographic conditions

The analysis was carried out using Shimadzu Prominence liquid chromatography equipped with LC-20AD quaternary solvent delivery system, auto-sampler having a universal loop injector of capacity 1-100 µL, and an SPD-M20A diode array detector monitored between 200 to 300 nm. A Windows-7 based LC- solution version 1.25. The column used was Thermo Hypersil-Gold C-18 column (150 mm × 4.6 mm i.d.; 5µm). The mobile phase was methanol: 2% acetic acid in a ratio of 50:50%, v/v (pH 5.10 ± 0.1, adjusted using acetic acid). The flow rate was 1.0 mL/min, and the injection volume was 10 µL. The detection wavelength was satisfactory at 300 nm that gives good response. The analysis time was below five minutes. Identification of timolol maleate signal was confirmed by UV spectrum, peak purity and retention times.

Preparation of standard solutions (calibration curve)

Different solutions of timolol maleate were prepared from stock solutions. 12.5, 25, 100, 200, 300, 400, 500 µL were pipetted into series of different 10 mL volumetric flasks. Aqueous methanol (50%) was added to achieve eight working solutions which are 1.25, 2.50, 5.0, 10.0, 20.0 and 40.0 µg/mL.

***In vitro* diffusion study**

This study was performed using automated Hanson vertical diffusion cell (VDC) equipment according to diffusion methods of FDA and USP to obtain diffusion profile rate of the drug from the ocusert. Each vertical cell capacity was 9 mL, and the rotation speed was 350 rpm. The test was performed over 24 h at 34.5 ± 0.5°C. The dissolution medium was phosphate buffer saline (PBS). In this test, the sampling was done at 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h by withdrawing 0.4 mL every stated time replacing by the same quantity of freshly prepared medium. The samples collected and analyzed by HPLC. *In vitro* release studies have been performed using PCP Disso v2.08 software (Poona College of Pharmacy, Pune, India) and appropriate release model was selected.

Drug–polymer compatibility study

Compatibility between drug and polymer, apart from its physical characteristics, is an important factor in determining the efficacy of poly-

mer delivery systems. The potential interaction between drugs and polymers was studied by Fourier transform infrared (FTIR) spectroscopy prior to final formulation using pure polymers, physical mixture of drug and polymers, uncross-linked ocusert, and cross-linked ocusert.

Physicochemical evaluation of the ocuserts

Physical characterization

The ocular inserts were evaluated for their physical characters such as color, texture, and appearance.

Drug content uniformity

Ocular insert belonging to each formula (M1-M17) was dissolved in suitable quantity of PBS using 45 MHz ultrasonic bath and solution was filtered using syringe filters (0.45 µm), suitably diluted and drug content was determined using Shimadzu Prominence liquid chromatography.

Uniformity of thickness

The thickness of ocuserts was determined using a Vernier caliper (Shanghai, China) and recorded as the mean of 20 measurements.

Swelling test

Three ocuserts of the selected formula were weighed and placed separately in Petri dishes containing 25 mL of phosphate-buffered saline (PBS, pH 7.4) solution. The dishes were stored at room temperature. The ocuserts were removed after 5, 10, 15, 20, 30, 45, and 60 min, wiped with tissue to remove excess PBS layer, weighed and then returned. The degree of fluid uptake was calculated using the equation [1] (Juliano et al., 2008):

$$\text{Swelling Index} = [(W_t - W_0)/W_0] \times 100 \quad [1]$$

where W_0 is the initial weight of the sample and W_t is its weight at time t.

In vivo studies

Ocular irritation

The selected ocusert formula was tested by positioning the ocuserts in the left eye's *cul-de-sac* of five male albino rabbits. Until diagnosis, both eyes of the rabbits were monitored for any redness, swelling, increased production of tears, or any signs of discomfort and studied up to 12 h (Kaur et al., 2000).

In vivo release study

The ocusert was inserted simultaneously in one eye of each of five rabbits and another eye was served as a control. After 24 h, the applied inserts were carefully removed and analyzed by HPLC to check the percent drug remained (Kumari et al., 2010).

In vivo therapeutic evaluation

In this study, Schiotz tonometer was used to record the initial IOP. The tested eyes (right eyes) of 18 rabbits were treated 3 times daily for 2 weeks by 0.5% dexamethasone eye drops while the left untreated eyes remain as a control. The intra ocular pressure (IOP) was measured twice a week at noon at specified time intervals and changes in IOP were recorded according to the scale, which was provided with the tonometer (Parmar and Tank, 2013).

The basic measurements were taken for both eyes for all animals. After inducing glaucoma in the right eyes of all rabbits, the animals were divided into three groups, each group contained six animals, the first group served as a control, the second group as a standard, which was treated with timolol eye drops and the third group treated with the ocuserts. The mean decrease in IOP profiles was calculated after the administration of timolol maleate (0.5%) eye drops once daily or application of timolol maleate ocusert in the *cul-de-sac* of the rabbit's eyes for 24 h as per protocol.

Stability study

The selected ocuserts were stored at different temperature conditions, which included refrigeration temperature (5 ± 0.5°C), room temperature (30 ± 0.5°C) and a temperature of (40 ± 0.5°C) for 3 months to evaluate the physical stability according to ICH guidelines (Nautiyal et al., 2012).

Statistical analysis

Statistical analyses of data were undertaken Design Expert 8.0.6 (Statease, USA) and MODDE 12.1 (Sartorius Inc., USA) software. Analysis of variance (ANOVA) was applied. $P<0.05$ was considered significant. Values are expressed as the mean ± standard deviation.

RESULTS

Design formulation of ocusert

The ocusert were designed using central composite design as per ICH guideline (ICH, 2004). Two-dimensional (2D) contour and perturbation plots were created to estimate the effect of independent factors (A, B or C) on drug loading and drug release. The least squares regression method was used to calculate the coefficients for the model. A linear relationship between drug loading, drug release and independent factor was observed after model reduction. It is evident from (Fig. 1A-B), that an increase in time (B) and temperature (C) at constant EDC concentration (A = 0.15) increases drug loading and drug releasing capacity. The scaled and centered coefficients are mentioned in the Fig. 1C, which indicates that time and temperature are the significant terms. Regressed equations for the drug loading [2] and drug release [3] are mentioned after the Table 1. Based on observations, observed responses, the formulation M-8 was selected as the desirable formulation and studied extensively.

$$\text{Drug loading} = 62.002 + (0.20927 \times \text{Time}) + (1.11915 \times \text{Temp}) \quad [2]$$

$$\text{Drug release} = 67.67 + (5.12 \times \text{Time}) + (4.52 \times \text{Temp}) \quad [3]$$

Evaluation of physical properties

Physical characterization

The ocuserts were visually inspected. Its texture was smooth, translucent, and the appearance was uniformed without any imperfections as shown in Fig. 2. In addition, their surface was homogeneous without any cracks or phase separation between the matrix and the drug. This would reflect a uniformed distribution of the ocusert components (drug and polymer). The physiochemical properties are presented in Table 1.

Drug content uniformity

The results indicate that the drug content in all batches was within the specified range. The drug content ranged from 96.2 ± 0.3% to 98.7 ± 0.5% as given in Table 1. This indicates that the method of preparation that was used has yielded reproducible results and that the drug has been uniformly distributed in the polymeric matrix (Bansal et al., 2013).

Uniformity of weight

The ocuserts were uniform and their weight was ranged from 9.1 ± 0.02 mg to 9.75 ± 0.02 mg. The low standard deviation and relative standard deviation reflects the weight uniformity of the ocuserts as shown in Table 1.

Uniformity of thickness

The prepared ocuserts were 6 ± 0.1 mm in diameter and thickness ranging from 0.35 mm to 0.3 mm with low standard deviation ± 0.02 values indicating the uniformity of the ocusert's thickness as shown in Table 1.

Percentage of moisture absorption

The water content of the selected formula (M8) was ranged from 2.99% to 3.29% (n = 3). While in case of wet ocuserts the moisture content was ranged from 78.83% to 77.71% (n = 3). Results of other formulas are illustrated in Table 1.

Table 1. Designed ocusert formulations (M1-M17) using central composite design, their physicochemical properties and observed responses.

F-ID	Type[1]	ECD (%) [Factor A]	Time (h) [Factor B]	Temp (°C) [Factor C]	Weight variation (mg)	Surface pH	Thickness (mm)	Moisture content (%)	Drug loaded (µg)	DR[2] (µg)	% DR	T_{max} (h)
M-1	F	0.10	24	4	9.47 ± 0.019	7.41 ± 0.05	0.32 ± 0.024	2.91 ± 0.22	68	54.0	79.4	2
M-2	F	0.20	24	4	9.55 ± 0.017	7.38 ± 0.04	0.33 ± 0.021	3.24 ± 0.19	70	55.0	78.6	2
M-3	F	0.10	72	4	9.46 ± 0.015	7.42 ± 0.03	0.31 ± 0.023	2.98 ± 0.26	90	75.2	83.7	1
M-4	F	0.20	72	4	9.52 ± 0.021	7.40 ± 0.04	0.33 ± 0.019	2.93 ± 0.17	68	54.1	79.6	4
M-5	F	0.10	24	12	9.69 ± 0.018	7.39 ± 0.07	0.35 ± 0.021	3.13 ± 0.18	81	65.5	80.9	2
M-6	F	0.20	24	12	9.38 ± 0.016	7.35 ± 0.05	0.33 ± 0.020	3.11 ± 0.24	80	68.3	85.4	4
M-7	F	0.10	72	12	9.22 ± 0.020	7.44 ± 0.03	0.31 ± 0.022	2.99 ± 0.19	83	69.7	84.0	2
M-8	F	0.002	72	12	9.15 ± 0.023	7.42 ± 0.04	0.3 ± 0.018	3.14 ± 0.15	98	82.5	84.1	2
M-9	A	0.07	48	8	9.37 ± 0.019	7.36 ± 0.08	0.32 ± 0.020	3.12 ± 0.18	71	58.0	81.7	2
M-10	A	0.23	48	8	9.68 ± 0.021	7.41 ± 0.06	0.35 ± 0.020	2.97 ± 0.16	84	70.0	83.3	2
M-11	A	0.15	7.64	8	9.31 ± 0.016	7.34± 0.03	0.33 ± 0.024	2.92 ± 0.21	76	61.5	80.9	2
M-12	A	0.15	88.36	8	9.71 ± 0.022	7.45 ± 0.07	0.35 ± 0.021	3.13 ± 0.15	93	80.0	86.0	8
M-13	A	0.15	48	1.27	9.64 ± 0.019	7.36 ± 0.02	0.34 ± 0.018	2.94 ± 0.20	76	62.0	81.6	2
M-14	A	0.15	48	14.73	9.41 ± 0.017	7.47± 0.08	0.32 ± 0.024	2.96 ± 0.16	85	70.35	82.8	2
M-15	C	0.15	48	8	9.75 ± 0.015	7.41 ± 0.01	0.35 ± 0.020	3.15 ± 0.18	89	77.0	86.5	4
M-16	C	0.15	48	8	9.75 ± 0.011	7.49 ± 0.06	0.34 ± 0.015	3.16 ± 0.11	80	65.98	82.5	4
M-17	C	0.15	48	8	9.70 ± 0.014	7.42 ± 0.04	0.34 ± 0.011	3.14 ± 0.15	85	68.01	80.0	4

The results are presented as mean ± SD. [1]Type: F: Factorial; A: Axial; C: Center points; [2]DR: Drug released.

Swelling test

The results indicated that the ocuserts belonging to M-8 formula had kept their integrity throughout the swelling studies and their swelling index was effectively controlled. The swelling behavior of the investigated ocusert is illustrated in Fig. 3. The ocuserts maintained their original oval shape in the swelling medium without any visible deformation throughout the swelling study. This observation was logically ascribed to the cross-linking efficiency of the gelatin polymer.

Drug-polymer compatibility study

The results showed that there is no chemical interaction between timolol maleate and the cross-linked gelatin, (1641 cm^{-1}) and (1517 cm^{-1}) peaks indicate the presence of new -CONH- bonds in the cross-linked polymer due to cross-linking of amino group (-NH_2) of gelatin group with carboxylic group (-COOH) groups as shown in Figs. 4-6).

High Performance Liquid Chromatography (HPLC)

The calibration curves of timolol maleate were constructed and the drug in formulation was determined using HPLC. The retention time of timolol maleate was consistent without any observation of drug-excipients interaction and new product/degraded products during analysis using HPLC-equipped with photo diode array detector. The calibration curve of pure timolol maleate was illustrated in Fig. 7.

***In vitro* drug release study**

According to the results mentioned it was observed that formulation M-8 was the best formulation because it is loaded successfully with high amount of drug amount, compared with other formulations (Table 1), the time required to release the maximum amount of drug within 2 hours. The release and kinetics studies of drug was found to be Koresmeyar Peppas diffusion model (R = 0.8186) indicating that the diffusion model gave a better and best fit model as the plots showed maximum linearity with R value (Amar et al., 2012). Furthermore, the 'n' value obtained from Koresmeyer Peppas model was (n = 0.2510). The results are illustrated in Fig. 8.

In vivo studies

In vivo release study

After 24 h, the ocusert was removed from the rabbit's eye and was analyzed using HPLC to estimate the drug remained or presence of unreleased drug, the result shows that the tested ocusert released the entire drug in 24 h. According to the above-mentioned results, the ocusert may act for 24 h (once daily), these results was in consistent with the *in vitro* release data.

In vivo study of tolerance and irritation

There is no sign of reflux blinking and redness in the animal's eyes and the animals were restful.

In vivo pharmacological evaluation

After 48 h the IOP was within the normal range in the animals that were treated with the ocuserts, the IOP in the eye drops treated group was 26.0 ± 0.45 mmHg. Thus, the timolol maleate ocusert reduces the IOP better than eye drops, this can be explained by that the ocusert keep the therapeutic level of timolol maleate for longer time and may overcome the rapid drainage and clearance of the eye drops from the eyes (Nair et al., 2015). The results are illustrated in Fig. 9A. After treatment for two days, the animals were left without treatment for three days. The IOP was measured and it was elevated again, which indicated that the reduction in IOP was due to effect of timolol, as shown in Fig. 9B.

Preliminary stability studies of selected ocusert (M-8)

The preliminary studies indicate that the ocuserts of selected formula (M-8) were stable with consistent appearance, up to 90 day at 30°C. There was slight change at higher temperature. The results are shown in Table 2.

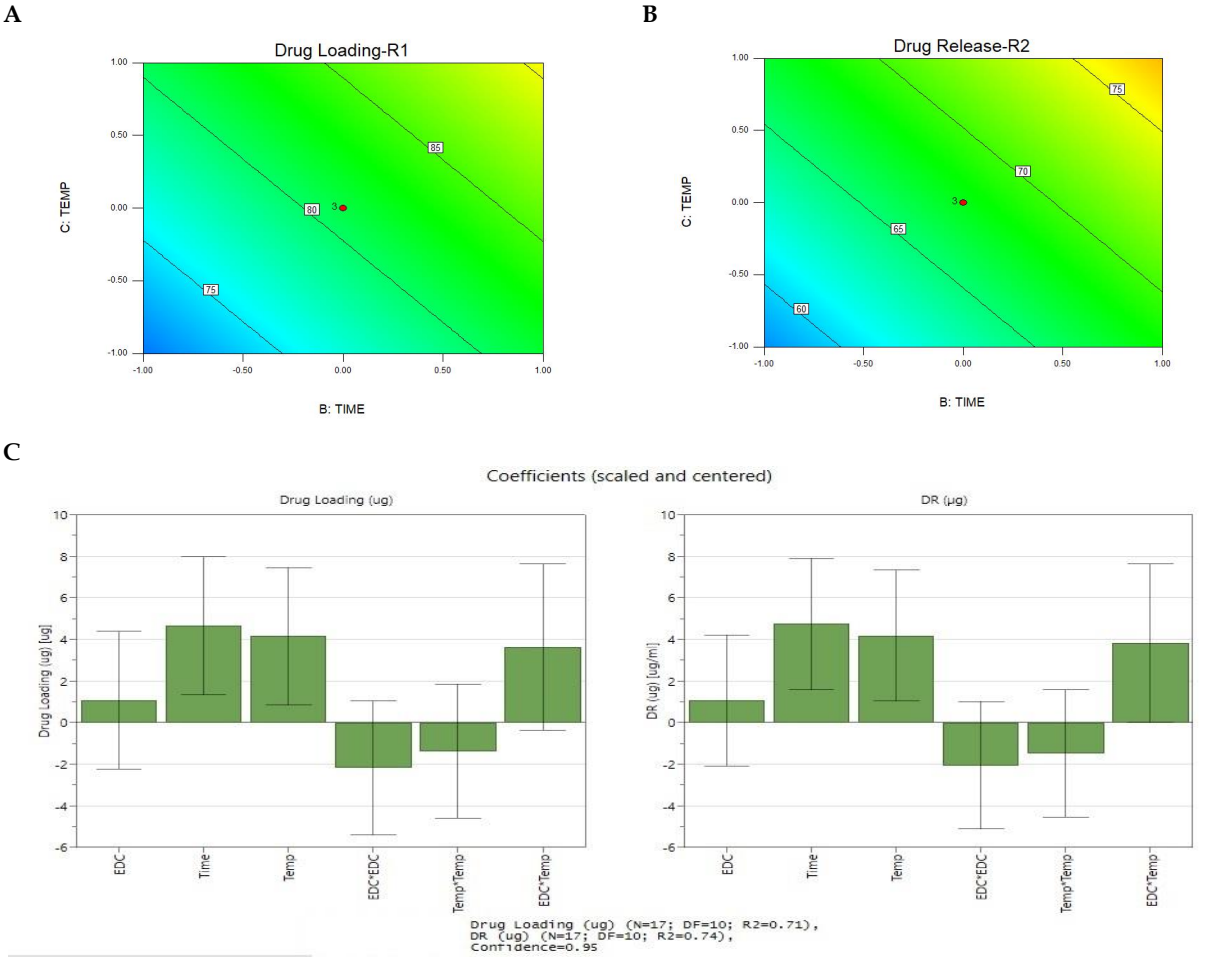

Figure 1. (A) 2-D contour plot for drug loading; **(B)** drug release *versus* independent factor (A-EDC, B-Time, C-Temperature); **(C)** scaled and centered coefficients for drug loading and drug release.

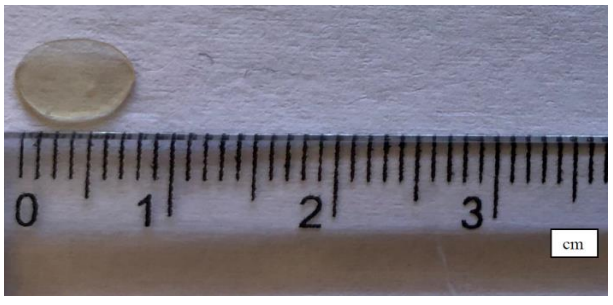

Figure 2. Ocusert shape and size.

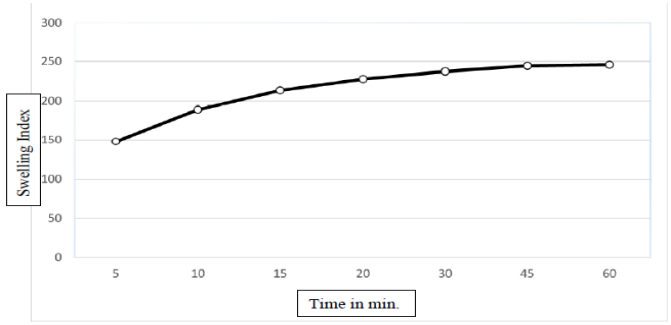

Figure 3. Swelling behavior of the investigated ocusert (M-8).

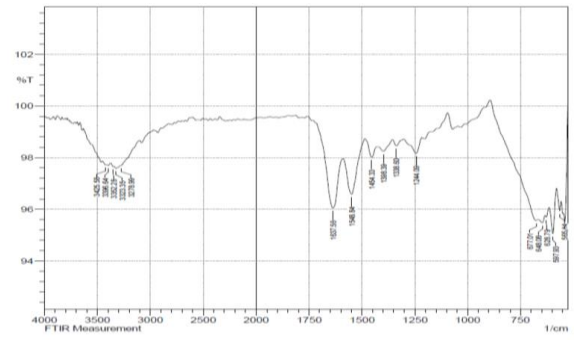

Figure 4. FTIR spectrum for non-cross-linked gelatin.

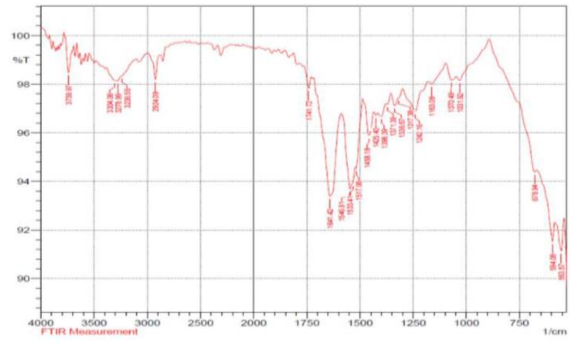

Figure 5. FTIR spectrum for cross-linked gelatin.

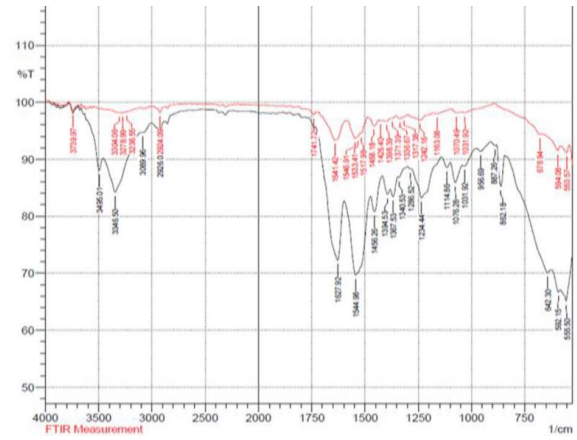

Figure 6. FTIR spectrum for cross-linked gelatin loaded with timolol maleate.

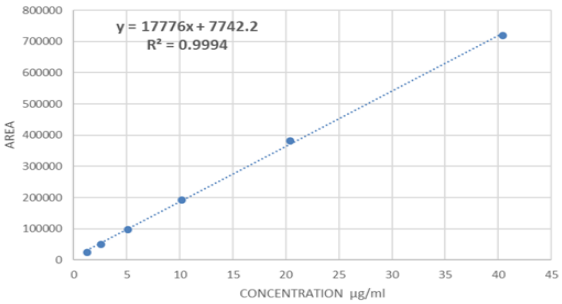

Figure 7. Calibration curve of timolol maleate using HPLC at 300 nm.

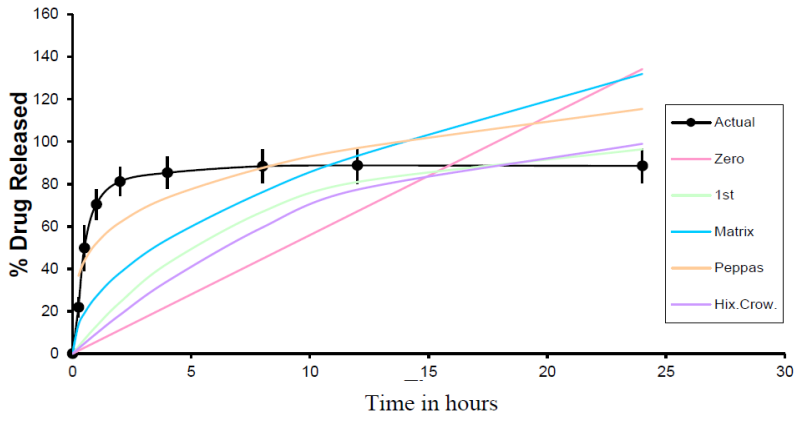

Figure 8. Release profile of selected ocusert formula (M-8).

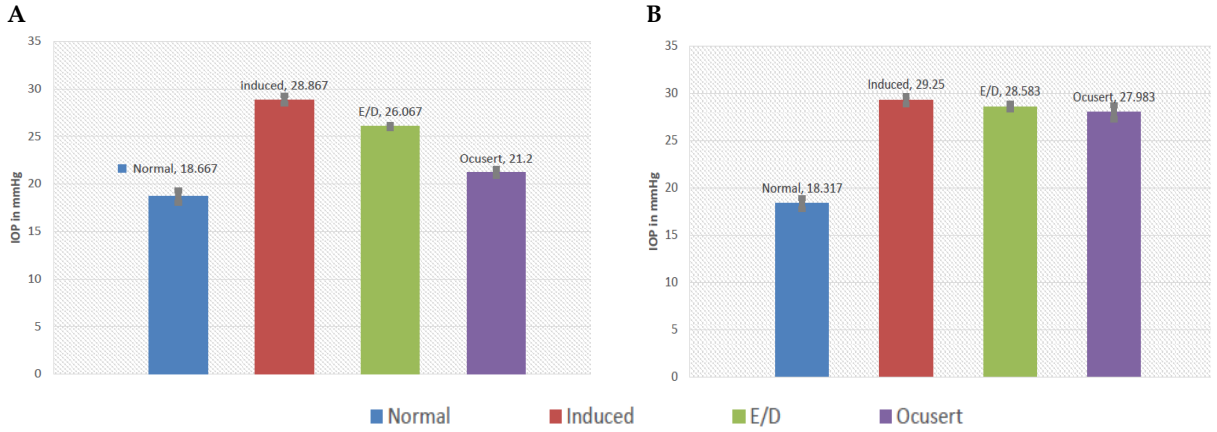

Figure 9. *In vivo* pharmacological evaluation. **(A)** IOP level between normal, ocusert (M-8), timolol E/D and untreated groups after 48 h treatment; **(B)** IOP level between normal, ocusert (M-8), timolol E/D and untreated groups (after discontinuation of treatment).

Table 2. Stability studies of selected ocusert (M-8).

Time (Days)	Appearance			
	At 5°C	At 30°C	At 40°C	At 60°C
0	Clear	Clear	Clear	Clear
15	Clear	Clear	Clear	Melt
30	Clear	Clear	Clear	Melt
60	Clear	Clear	Turbid	Melt
90	Clear	Clear	Sticky	Melt

DISCUSSION

In this study, timolol maleate ocuserts were formulated using gelatin cross-linked with EDC and NHS such cross-linkage has been conducted previously (Kuijpers et al., 2000). The cross-linkage between gelatin, and the incorporation of timolol in the gelatin polymer was confirmed by FTIR. Seventeen different formulas were prepared using the Central Composite Design (Box and Wilson, 1992) by solvent casting method (Lai et al., 2006) and the best formula was selected and evaluated extensively. The physical properties of the selected ocusert formula were studied and these were found to meet all the requirements for ophthalmic ocuserts. The physical properties of the prepared ocuserts were studied including the evaluation of weight, thickness, percentage of moisture absorption, drug content and swelling test as mentioned in previous researches. The release studies of the ocuserts of selected formula were evaluated extensively. The release of timolol from the selected formula (M-8) was evaluated *in vitro* using automated diffusion cell, the release pattern was found to follow the Koresmeyer-Peppas model while the *in vivo* study was conducted by measuring the decrease in the intraocular pressure in dexamethasone induced glaucoma rabbit's eyes (Parmar and Tank, 2013).

As shown in the results, the prepared ocusert intended for once daily use decreases the IOP for twenty-four hours, the cornea and the conjunctiva of rabbit's eyes show an excellent tolerability after ocusert insertion without observing irritation or redness. The physical properties of ocusert and the *in vivo* release of the drug were not influenced af-

ter insertion inside the *cul-de-sac*. The sustained decrease in IOP by the prepared ocusert is important for the therapeutic effect of timolol maleate in treatment of glaucoma. Long term stability studies are in progress.

CONCLUSIONS

In this study, timolol maleate ocuserts in cross-linked gelatin polymer were formulated. The *in vitro* release studies indicate that the maximum release of timolol maleate occurred at two hours and more than 95% of the drug was released in twenty-four hours. The pharmacological effect of the selected ocusert formula was evaluated by measuring the decrease in IOP in experimental rabbits. There was a correlation between *in vivo* and *in vitro* release of timolol maleate, which is associated with a decrease in IOP in experimental rabbits.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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AUTHOR CONTRIBUTION:

Contribution	Kanaan MQ	Numan NA	Shakya AK	Tawfiq FA
Concepts or ideas			x	x
Design			x	
Definition of intellectual content		x	x	x
Literature search	x		x	
Experimental studies	x	x	x	x
Data acquisition	x	x	x	x
Data analysis	x	x	x	
Statistical analysis	x	x	x	x
Manuscript preparation	x	x	x	x
Manuscript editing	x	x	x	x
Manuscript review	x	x	x	x

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