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Research Paper

Reduction of intraocular pressure using timolol orally dissolving strips in the treatment of induced primary open-angle glaucoma in rabbits

Yasmin A. El-Feky[a], Dalia A. Mostafa[b], Majid M. Al-Sawahli[c], Rania Farag A. El-Telbany[d], Sherin Zakaria[e], Ahmed M. Fayez[f], Kawkab A. Ahmed[g], Ebtesam M. Alolayan[h] and Dalia Farag A. El-Telbany[a]

[a]Department of Pharmaceutics, Faculty of Pharmacy, Modern University for Technology and Information (MTI), Cairo, [b]Department of Pharmaceutics, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), Giza, [c]Department of Pharmaceutical Technology, Faculty of Pharmacy, Kafr Elsheikh University, Kafr Elsheikh, [d]Department of Biochemistry, Faculty of Pharmacy, Modern University for Technology and Information (MTI), Cairo, [e]Department of Pharmacology and Toxicology, Kafr Elsheikh University, Kafr Elsheikh, [f]Department of Pharmacology and Toxicology, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), [g]Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt and [h]Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia

Keywords
fast release; intraocular pressure; orally dissolving strips; primary open-angle glaucoma; timolol

Correspondence
Majid M. Al-Sawahli, Department of Pharmaceutical Technology, Faculty of Pharmacy, Kafrelsheikh University, Kafrelsheikh, 33516, Egypt.
E-mail: majidalsawahli@gmail.com

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Abstract

Objective To enhance bioavailability of timolol (TML) and utilize alternatives for traditional eye drops for more patient compliance, this study was aiming to develop biodegradable orally dissolving strips (ODSs) of TML for treatment of primary open-angle glaucoma (POAG).

Methods Novel ODSs of TML were formulated and optimized using solvent casting method according to full factorial design ($3^1.2^2$). TML ODSs were characterized with respect to many parameters. *In-vivo* test was carried out using four groups of 24 New Zealand albino rabbits. POAG was induced by subconjunctival treatment of betamethasone. Histopathological examination and oxidative stress markers assay were carried out.

Key findings The optimized formula (F9) exhibited a remarkably 15-s disintegration time and 96% dissolution rate after 10 min. The results revealed a potent significant inhibitory effect of the optimized TML ODS to reduce IOP in induced rabbits in comparison with control rabbits and TML eye drops-treated rabbits. The formula showed also high activity against oxidative stress and absence of histopathological changes in iridocorneal angle and cornea.

Conclusion The ODSs could be a promising alternative delivery system for eye drops with more compliance to enhance delivery and therapeutic activity of TML in treatment of POAG.

Introduction

The World Health Organization (WHO) reported that total population in 2017 was around 7.5 billion, of which 36 million people suffer from blindness.[1] Glaucoma is a progressive optic neuropathy that results in irreversible blindness, and glaucoma affects more than 70 million people worldwide and is considered a public health crisis.[2] Primary open-angle glaucoma (POAG) is one of the leading causes of blindness in the United States and worldwide. Three to six million people in the United States are at increased risk for developing POAG because of elevated intraocular pressure (IOP) or ocular hypertension.[2] The elevation of IOP is the most important risk for glaucoma; hence, lowering of IOP remains the only proven therapy to slow the progression of vision loss in POAG.[3] Results from several clinical trials have demonstrated the benefit of lowering intraocular pressure in preventing the development and slowing the disease's progression.[4,5] There were also other factors which may contribute to optic nerve damage caused by glaucoma; as insufficient ocular blood flow leading to optic nerve ischaemia and glaucomatous

optic neuropathy, this hypothesis was evidenced in both animal and human studies,[6–8] where decreased ocular perfusion in animals can induce the retinal ganglion cell loss in spite of a normal IOP.[9,10]

Although the spread of eye drops for their multiple benefits and advantages, patient non-compliance is a critical factor leads to treatment failure.[11] The bioavailability of traditional ocular drug delivery systems such as eye drops is very poor because eye is protected by a series of complex defence mechanisms that make it difficult to achieve an effective drug concentration within the target area of the eye.[12] Also, many factors affect the resultant bioavailability like nasolachrymal drainage, lacrimation, drug dilution with tear fluid, tear turnover and conjunctival absorption.[13,14] Referring to the silence and chronic nature of POAG, it is recommended to offer alternative delivery systems that achieve sufficient and persistent compliance aiming to reduce IOP.

Timolol maleate (TML) is a non-selective beta-blocking drug used for the treatment of increased intraocular pressure in patients with chronic open-angle glaucoma.[15] US Food and Drug Administration (FDA) considered TML as the 'gold standard' drug for IOP reduction since its approval in 1979 for ophthalmic use.[16] Due to its short elimination half-life (4 h), it is orally administered twice daily. Additionally, because of low bioavailability (50%), a high oral dose of 10–60 mg/day was required. As an adverse effect, bronchospasm was reported in some patients.[17,18]

Orally dissolving strips (ODSs) are unique and robust delivery systems, and they have the potential of simplicity of preparation, easiness of oral handling without need for water, fast delivery from swift disintegration, delivering to systemic circulation directly after buccal or sublingual administration, avoiding first-pass metabolism and improved bioavailability.[19,20] From other point of view, it is interesting to note that the permeability of buccal mucosa is approximately 4–4000 times greater than that of the skin. Also, the oral mucosa is highly perfused with blood vessels. Hence, therapeutic serum concentrations of a drug can be achieved rapidly.[21] Based on these facts, it is very attractive to utilize the dual benefits of both ODS and buccal/sublingual administration.

As it has been reported that there is a significant relation between lowering diastolic blood pressure and diastolic perfusion pressure with open-angle glaucoma,[22] this work was aiming to develop and investigate TML ODSs which may immediately lower the blood pressure by rapid absorption of TML released after complete dissolving of the ODSs; also, high levels of TML can enhance blood supply to eye in addition to decreasing IOP.

Materials and Methods

Materials

Timolol maleate was purchased from (Merck, Darmstadt, Germany). Hydroxypropyl methylcellulose E15 (HPMC E15) was purchased from Sigma-Aldrich, St Louis, MO, USA. Polyvinyl pyrrolidone K30 (PVP K30) and chitosan were purchased from Fluka AG, Buchs, Switzerland. Polyethylene glycol 400 (PEG 400) was purchased from Prolabo, Paris, France. Betamethasone dipropionate/sodium phosphate salts were purchased from Medical Union Pharmaceuticals, Ismailia, Egypt. Timolol maleate 0.5% eye drops and oxybuprocaine hydrochloride and 0.4% sterile ophthalmic solution were purchased from Egyptian International Pharmaceutical Industries Co., 10th Ramadan city, Egypt. All other chemicals and solvents were of analytical grade.

Fourier transform-infrared spectroscopy analysis

A compatibility study using Fourier transform-infrared spectroscopy (FTIR) analysis was carried by IRAffinity-1, Schimadzu and Japan Spectrometer to investigate the possible interactions between pure TML and incorporated excipients which will be used in the formulation of ODSs. The FTIR spectrum was done for each of TML, HPMC E15, PVP K30 and chitosan, in addition to a physical mixture of the drug and each polymer in the ratio of 1 : 1. Each sample was separately mixed with KBr (IR grade), and the samples were directly scanned over a wave number range of 4500–500 cm^{-1}, as mean of three determinations.

Factorial design

A full factorial design ($3^1.2^2$) was constructed in this study using Design expert 8.0.7 software (StatEase Inc., Minneapolis, MN, USA). The studied factors were the plasticizer type (X_A), the polymer concentration (X_B) and the polymer type (X_C). Levels for each variable were determined (Table 1). The design was aimed to study the combined effect of these factors on disintegration

Table 1 Full factorial ($3^1.2^2$) design for the preparation of TML ODSs

Factors	Levels		
X_A[a]	PEG 400	Glycerine	
X_B[b]	250	400	
X_C[c]	Chitosan	PVP K30	HPMC E15

[a]X_A, plasticizer type. [b]X_B, polymer concentration. [c]X_C, polymer type.

time (DT; Y_1) and per cent amount of drug dissolved after 5 min ($\%Q_{5min}$; Y_2). Twelve experimental runs were prepared; each run was carried out in triplicate.

Preparation of TML ODSs

Orally dissolving strips containing 10 mg of TML were prepared by solvent casting method. Polymer was dissolved in 10 ml distilled water and maintained at 50 °C till completely solubilized. TML, 0.05 mg sucrose and 10 mg citric acid were then dissolved. Plasticizer was later added. Homogenous solution was prepared using magnetic stirrer (SB162, Heidolph Brinkmann, Schwabach, Germany) for 10 min, then poured in silicone cupcake moulds and left to dry for at least 24 h. The moulds were checked for any imperfections and cut into the required size (2 × 2 cm^2) to deliver the equivalent dose of 10 mg per strip, then packed immediately in individual airtight aluminium seal packs and stored at 25 °C until use. Furthermore, visual inspection of all strips was performed; the prepared strips were checked for surface perfection, smoothness and ease of removal from silicone cupcake without rupturing, folding or cracking. The composition of the prepared ODSs is shown in Table 2.

Characterization of TML ODSs

Weight variation

Individual strips of 2 × 2 cm were weighed, and the average weights were calculated. Then, the average weight of the strips is subtracted from the individual weight of the strips.[23,24] Each test was carried out in triplicate.

Strip thickness

Thickness was measured by using calibrated digital vernier calipers (Mitutoyo, Kawasaki, Japan). The thickness of each strip was measured at five different locations (centre and four corners).[25] Data were represented as a mean ± SD and determinations.

Folding endurance

The strip is rolled or folded for many times until it breaks, and this determines the endurance of the strip. As the times of folding increase the endurance of strip increases. It was a directly proportional relation.[23,24]

Drug content

Each oral strip was dissolved in 10 ml Sorensen's phosphate buffer (pH 6.8). The absorbance of the solution was then measured spectrophotometrically at 294 nm using UV spectrophotometer (UV-1700; Shimadzu, Kyoto, Japan). The test was done on three strips.[26]

Surface pH

Six strips were taken randomly from different parts of the film. Each strip was moistened with 0.5 ml of double-distilled water and kept for 2 min. The pH was measured by touching the electrode of a pH meter (Jenway 3510, Swedesboro, NJ, USA) with the surface of the moistened film. The average values of readings for each strip are reported.[27]

In-vitro disintegration time

The test was carried using USP disintegration apparatus (Electrolab, Mumbai) using Sorensen's phosphate buffer at 37 ± 0.5 °C to simulate saliva in the test.[28,29] The time required for the strip to break was noted as *in-vitro* disintegration time.[30] The test was performed triplicate and mean ± SD was calculated.

In-vitro dissolution test

Dissolution test was carried out using USP type-1 (basket-type) dissolution apparatus (Pharma Test, Type PTW,

Table 2 Composition of timolol orally dissolving strips

Ingredients (mg)	Formulation code											
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
TM	10	10	10	10	10	10	10	10	10	10	10	10
HPMC E15	–	–	–	–	–	–	–	–	250	250	400	400
Chitosan	250	250	400	400	–	–	–	–	–	–	–	–
PVP k30	–	–	–	–	250	250	400	400	–	–	–	–
PEG 400	250	–	250	–	250	–	250	–	250	–	250	–
Glycerine	–	250	–	250	–	250	–	250	–	250	–	250
Flav. pineapple (ml)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Water	10	10	10	10	10	10	10	10	10	10	10	10

Germany). It was carried out in 500 ml of Sorensen's phosphate buffer (pH 6.8) maintained at 37 ± 0.5 °C and 50 rpm.[24] Aliquots each of 5 ml were taken at time intervals from 1 to 30 min. TML concentration in the samples was then determined spectrophotometrically at λ_{max} 294 nm against calibration curves.

Stability study

The optimized formula was subjected to investigate storage effect at high temperature and humidity (40 °C ± 2 °C/ 75%RH ± 5%RH). The sample was packed as individual strips in aluminium foil in stability chamber for 6 months. The sample was subjected again for any change in appearance, thickness, surface pH, weight variations, drug content, folding endurance, and disintegration time and drug dissolution.[31]

In-vivo study and measurement of IOP

Animals. Twenty-four New Zealand albino rabbits weighing 1.5–2.0 kg, purchased from research institute of ophthalmology (Giza, Cairo, Egypt), were used in the current study. Rabbits were housed individually in a pathogen-free facility. The facility was maintained at [25 ± 0.5] °C with (55 ± 1%) relative humidity and a 12-h light: dark cycle. All rabbits had ad libitum access to standard rodent chow and filtered water. Animals were left for 1 week before the start of the study for adaptation. General and ophthalmic examinations of all selected animals before the beginning of the study confirmed normal findings. Animals were observed for any irritation or inflammation in their eyes after drug administration. The study protocol and animals use were approved by The Ethical Committee of Faculty of Pharmacy, Kafr Elshiekh University. The experimental procedures were done in comply with the ARRIVE guidelines in accordance with the U.K. Animals (Scientific Procedures) Act, 1986, and associated guidelines, EU Directive 2010/63/ EU for animal experiments.

Rabbits were randomly assigned to four groups (six rabbit each) as shown in Figure 1. One group of rabbits were left as controls and treated with saline eye drops for 14 consecutive days. Glaucoma was induced in all groups other than control ones by a single subconjunctival dose of betamethasone, 0.05 ml of betamethasone in the right eye (each 1 ml contain betamethasone dipropionate 5 mg and betamethasone sodium phosphate 2 mg) and reserved under observation for 7 days.[32] At the 7th day, the second group of rabbits was treated with 0.05 ml of TML maleate eye drops (0.5%) in the right eye daily for further seven consecutive days (TML group). TML ODSs (10 mg/kg per day) were given to the third group of rabbits via oral gavage starting from the 7th to the 14th day of treatment (TML ODSs group). The fourth group of rabbits was left without any additional treatment for further 7 days (betamethasone group). The drug was prepared at concentration 7 mg/ml. Each 1 ml fitted to contain 5 mg betamethasone dipropionate and 2 mg betamethasone sodium phosphate.

All rabbits were adapted over several days for IOP measurement before the beginning of the study. The IOP was measured according to the previously reported method by Smriti[27] The animals' eyes were first disinfected with ethanol and then anaesthetized with one drop of oxybuprocaine hydrochloride 0.4% sterile ophthalmic solution. One minute later, the eyes were checked on a hard convex surface. The tonometer (RiesterSchiötz C, Jungingen, Germany) was then placed vertically on the cornea of the laterally positioned animal, carefully the eye lids held apart with one hand, being carefully not to press on the eye ball to avoid

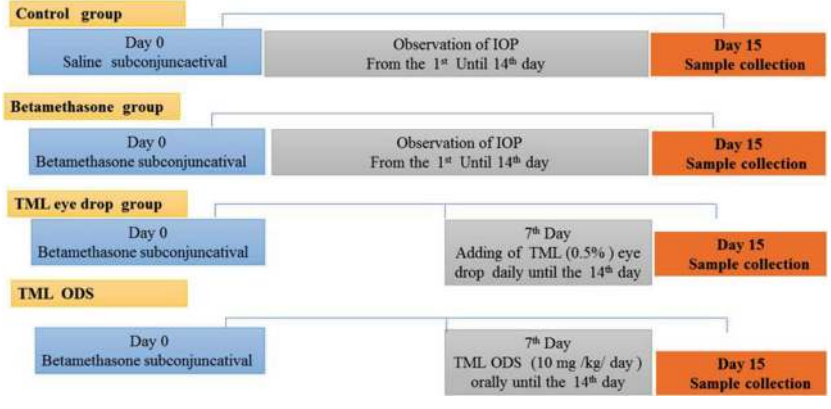

Figure 1 *In-vivo* study design. [Colour figure can be viewed at wileyonlinelibrary.com]

any error in the measurement of the IOP. The reading on the calibrated scale was noted and converted to mmHg from the converting table supplied with the tonometer. Three IOP readings were taken, and data are presented as the average of the three reading. Measurements of IOP was always started at the same time of the day to avoid daily variations of IOP.[33]

Blood samples collection. For collecting blood samples, rabbits were anesthetized via intraperitoneal administration of sodium pentobarbital at 30–40 mg/kg. Blood samples were immediately obtained from jugular vein. Each blood sample was divided into two aliquots: the first one was added to EDTA-coated tube, while the other one was added to heparinized tube. The samples were then centrifuged (3000*g*, 10 min, 4 °C), and the resultant plasma in each supernatant was recovered and stored at 20 °C until analysis. Plasma recovered from EDTA-coated tubes was used for reduced glutathione (GSH) assay where the heparinized ones were used for malondialdehyde (MDA) assay.

Histopathological examination and histomorphometric analysis. For preparation of tissues, anesthetized animals were killed by cervical dislocation. Eyes were collected and fixed in 10% neutral-buffered formalin, washed, dehydrated in alcohol, cleared in xylene and embedded in paraffin. Paraffin blocks were sectioned at 4–5 μm and stained with haematoxylin and eosin for routine histopathological examination.[34] Histomorphometric analysis of the total retinal thickness was measured for five microscopic fields/cross sections ($n = 6$) from each group by a light microscope (Olympus BX50, Tokyo, Japan) under high-power magnification (×400) using TS View version 6.2.4.5 software.

Assay of oxidative stress markers. Oxidative stress was assayed via determination of MDA and GSH using kits (Biodiagnostics, Giza, Cairo, Egypt). The designed protocols were prepared based on assays described in Ref. [35,36].

Statistical analysis. All values are presented as means ± SD. Comparison between groups was estimated using one-way analysis of variance (ANOVA) with Tukey's test as post hoc test. P values <0.05 were considered significant. Statistical analyses were performed using Prism software (v.6.0; GraphPad, San Diego, CA, USA).

Results and Discussion

FTIR analysis

Fourier transform infrared spectroscopy spectra of pure TML, individual polymer and physical mixture of drug with different utilized polymers are shown in Figures 2–4. FTIR spectrum of pure TML (Figure 2) showed the bands assigned to N-H stretching and OH stretching appeared in the region of 3289 and 3135 cm^{-1}, respectively. CH stretching and CH bending were observed at 1375 and 2968 cm^{-1}, 1706 cm^{-1} correspond to C=O stretching vibration, 1583.32 and 1448.66 cm^{-1} correspond to C=C aromatic rings, and this spectrum was in accordance with Refs. [37–39]. HPMC E15 exhibited major bands as illustrated in Figure 3a at 3464 and 3448 cm^{-1} for O–H stretching band of alcohol; 2939, 2904 and 2835 cm^{-1} for –CH stretching of alkane; 1639 cm^{-1} for C=C, 1458 cm^{-1} for C=C aromatic; 1377 cm^{-1} for –C–H– alkane; 1319 cm^{-1} for C–O ether; 1265 cm^{-1}, 1122 and 1064 cm^{-1} for C–F stretching band of alkyl halide; and 945 cm^{-1} for =C–H bending for alkenes. The major bands of PVP K30 as in Figure 3b appeared at 3434 cm^{-1} for O–H stretching band of alcohol and 1651 cm^{-1} for C=O carbonyl group. Chitosan exhibited major bands at 3437 cm^{-1} for O–H stretching band of alcohol and amine group NH_2 as in Figure 3c. The comparative spectra of the three mixtures of drug and polymers in ratio 1 : 1 showed that the major bands

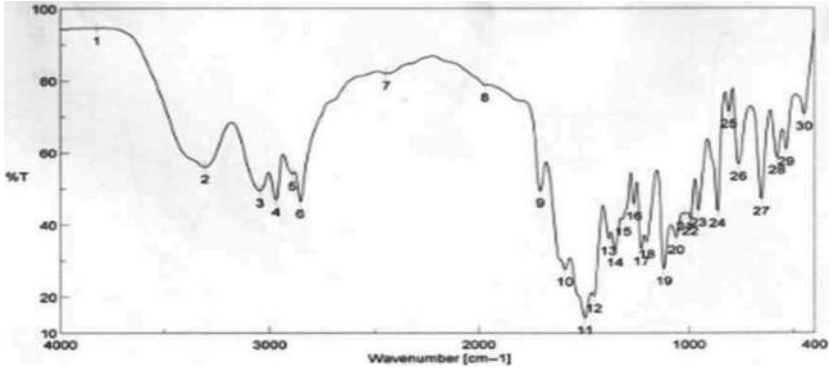

Figure 2 Fourier transform-infrared spectroscopy spectra of pure timolol.

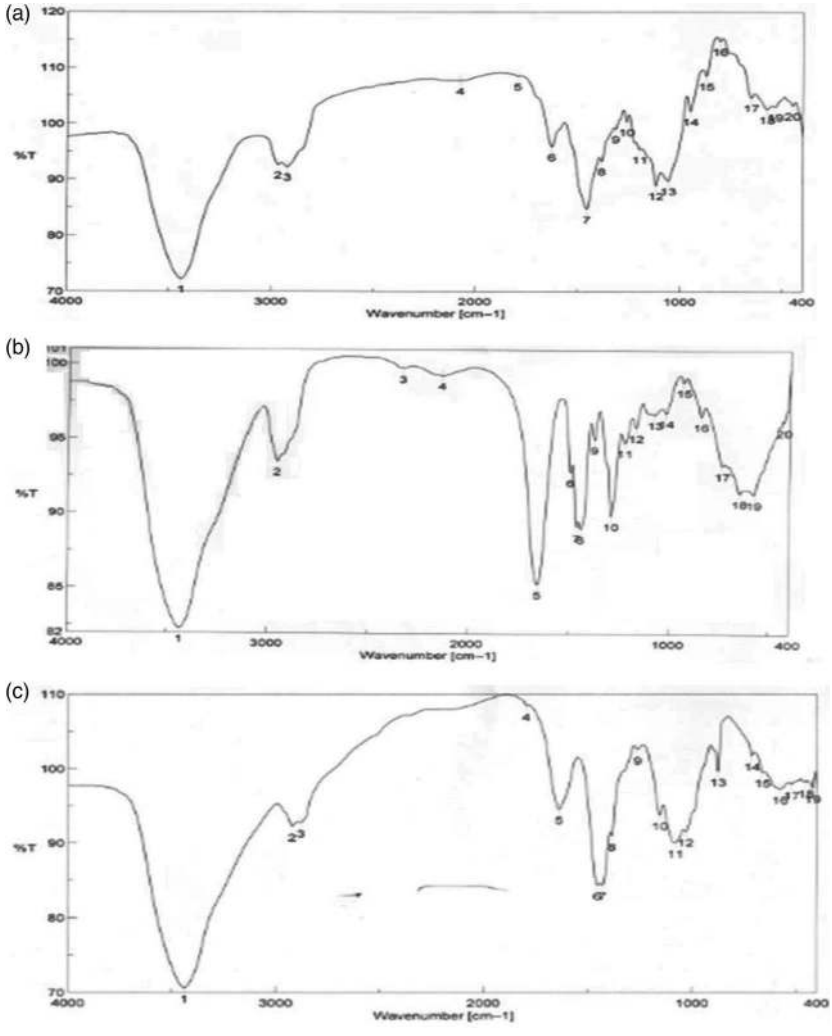

Figure 3 Fourier transform-infrared spectroscopy spectra of (a) HPMC E15, (b) PVP K30 and (c) chitosan.

of TML are located exactly in the same[35] positions (Figure 4); consequently, there is no movement of the peaks means there was no interaction occurs. This confirms the absence of any incompatibility between the TML ODSs ingredients.

Preparation of TML ODSs

Solvent casting method was utilized to prepare twelve formulae of TML ODSs. HPMC E15, PVP K30 and chitosan were used as water soluble polymers as strip former. Glycerine and PEG 400 was used as plasticizers. Sucrose was used as sweetening agent. Citric acid was used as saliva-stimulating agent to promote ODS disintegration in oral cavity.[36,37] The obtained ODSs were transparent, homogenous, dry, flexible and thin without any air pubbles.

Characterization of TML ODSs

Weight variation

As shown in Table 3, the prepared ODSs were found to be varied in weight from 21.36 ± 0.45 mg to 24.66 ± 1.5 mg. The lake of any large variation in weight indicates the efficiency of the method employed and was likely to have uniform drug distribution.

Strip thickness

The thickness of different prepared strips ranged from 20 ± 0.42 to 25.1 ± 0.56 μm (Table 3). Strip thickness measurement was essential to ascertain the uniformity of the strip thickness as it was directly related

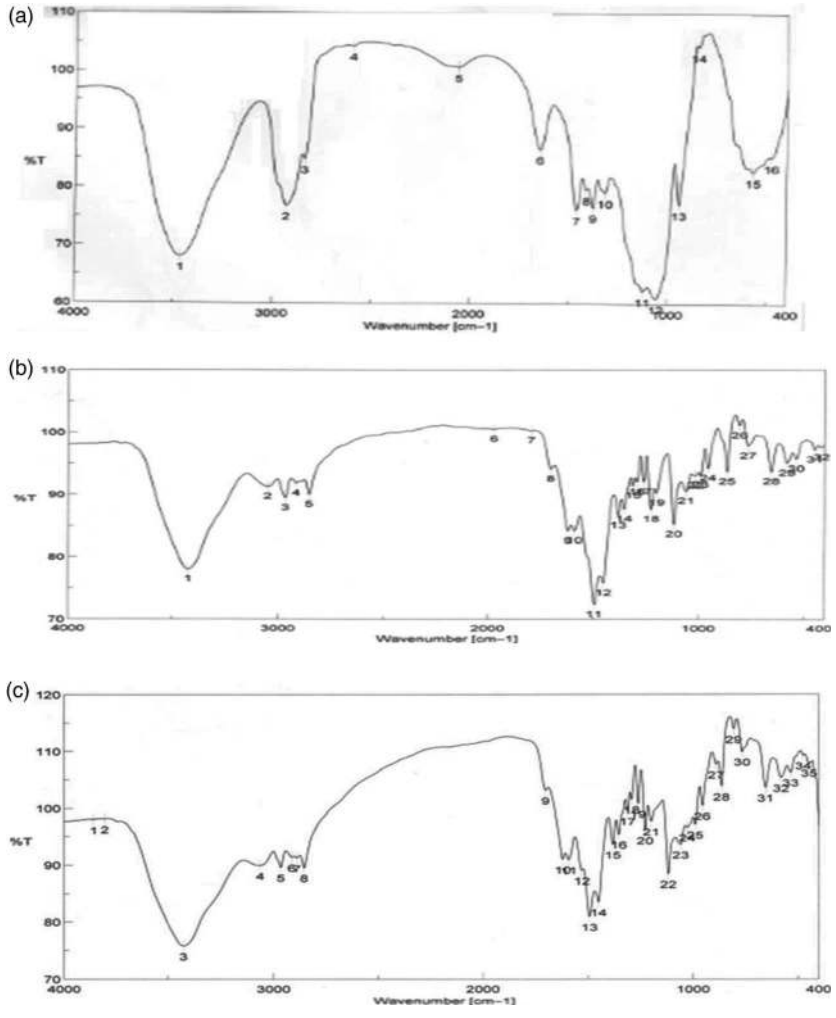

Figure 4 Fourier transform-infrared spectroscopy spectra of physical mixtures of timolol with (a) HPMC E15, (b) PVP K30 and (c) chitosan.

Table 3 Physicochemical characterization of the prepared timolol orally dissolving strips

Formulae	Weight variation (mg)	Thickness (μm)	Folding endurance	Drug content (%)	Surface pH	DT (s)
F1	24.61 ± 1.5	20 ± 0.42	112 ± 1.34	98 ± 1.6	6.6	285 ± 1.46
F2	24.13 ± 0.6	20.4 ± 0.54	109 ± 0.56	95 ± 0.74	6.6	310 ± 0.53
F3	23.94 ± 0.3	21 ± 0.51	112 ± 0.59	98 ± 2.54	7	371 ± 0.15
F4	24.11 ± 0.4	21.7 ± 0.62	99 ± 0.25	96 ± 1.25	7	440 ± 0.88
F5	23.46 ± 1.11	23 ± 0.57	280 ± 0.11	95 ± 2.5	7	66 ± 0.65
F6	23.59 ± 0.5	24 ± 1.56	291 ± 1.27	94 ± 3.4	6.9	78 ± 0.49
F7	24.13 ± 0.17	22.2 ± 0.88	300 ± 1.51	95 ± 1.6	6.8	81 ± 0.23
F8	24.16 ± 0.27	22.9 ± 0.79	286 ± 0.65	95 ± 0.7	6.9	90 ± 0.38
F9	24.49 ± 1.5	25 ± 0.22	156 ± 0.79	97 ± 0.44	7	7 ± 0.42
F10	21.36 ± 0.45	24.8 ± 0.33	172 ± 0.52	93 ± 0.59	7	13 ± 0.27
F11	22.56 ± 0.69	25.1 ± 0.56	164 ± 0.61	94 ± 0.56	7	17 ± 0.15
F12	21.91 ± 0.37	24 ± 0.46	169 ± 0.52	93 ± 0.5	6.8	24 ± 0.36

to the dose accuracy. In general, an ideal oral strip should exhibit a thickness between 50 and 1000 μm. All the prepared strips contain different amount of polymers; hence, the thickness was gradually increased in accordance with the increase in polymers amount.[24,38,39]

Folding endurance

The flexibility of strips was an important physical character needed for easy application on the site of administration.[24] Strip formulae exhibited good folding endurance which indicates good flexibility. The results and comparative folding endurance of all formulae were in the range of 99 ± 0.25–300 ± 1.51 (Table 3). The high value of folding endurance is an indication to high elasticity of the prepared ODSs possibly due to incorporation of plasticizer.

Drug content

TML content was found to be ranged from 93 ± 0.59 to 99 ± 3.4 (Table 3). It was observed that all the prepared strips were satisfactory in uniformity of drug and ensuring drug uniformity in each strip.

Surface pH

The surface pH of strips should be close to neutral pH comparable to buccal cavity pH. It was found to be in the range of 6.6–7 (Table 3), which ensure the absence of any irritation to the mucosal lining of the oral cavity due to possible alkalinity or acidity.

In-vitro disintegration time

Disintegration time represents an indication to drug onset of action desired for ODSs formulae. The mean time for complete disintegration of ODSs varied from 7 ± 0.42 to 440 ± 0.88 s (Table 3). The lowest values of DT allow faster drug release and faster oromucosal absorption. Also according to Tedesco, Jyoti, they could be classified as flash release strips.[29,40] Rapid disintegration of the strips was facilitated by their thinness (28–80 μm). Although there were no official guidelines to the oral strip, the DT will differ depending on the formulae. The DT limit of 30 s or less for orally disintegrating tablet described in Centre for Drug Evaluation and Research (CDER).[41] Guidance can be applied to fast dissolving oral strips; disintegration time will vary depending on the formulae but typically the disintegration range from 5 to 30 s. CDER describes tablets that take longer than 30 s to disintegrate may be appropriately considered to be chewable or oral tablet not described as fast disintegrating.

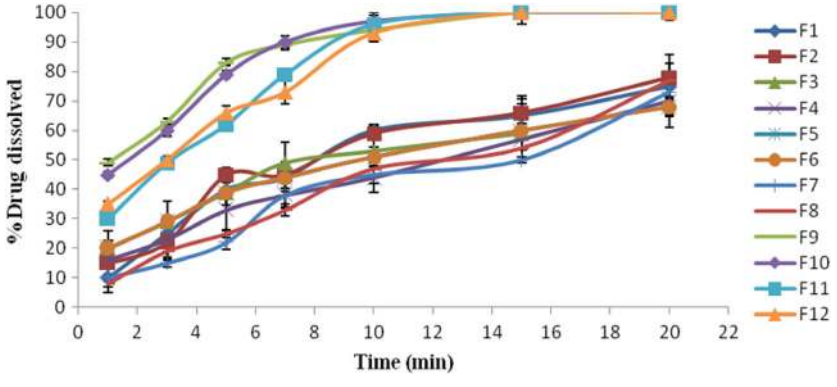

Figure 5 Dissolution profiles of timolol from the prepared orally dissolving strips. [Colour figure can be viewed at wileyonlinelibrary.com]

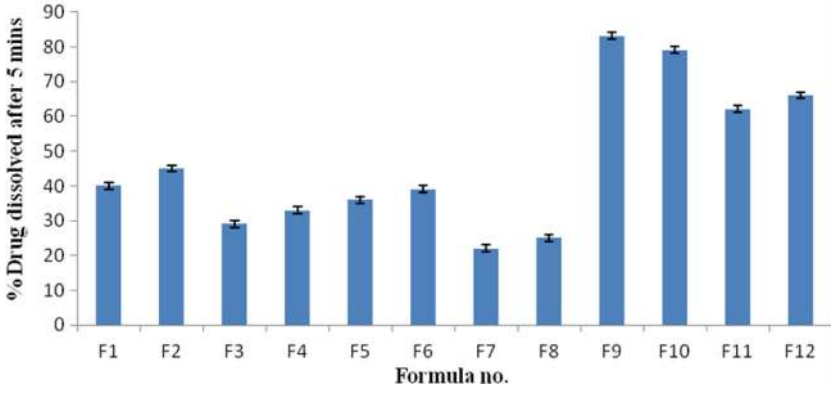

Figure 6 $\%Q_{5min}$ of timolol from orally dissolving strips. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 4 ANOVA for selected factorial model

Source	Sum-of-squares	df	Mean square	*F*-value	*P*-value
DT					
X_A	3844.00	1	3844.00	61.07	<0.0001
X_B	3721.00	1	3721.00	59.12	<0.0001
X_C	6.057E + 005	2	3.028E + 005	4811.44	<0.0001
Residual	1510.56	24	62.94		
R^2	0.9976				
Adj R^2	0.9967				
Pred R^2	0.9945				
$\%Q_{5min}$					
X_A	113.78	1	113.78	26.21	<0.0001
X_B	2177.78	1	2177.78	501.76	<0.0001
X_C	13621.56	2	6810.78	1569.20	<0.0001
Residual	104.17	24	4.34		
R^2	0.9936				
Adj R^2	0.9911				
Pred R^2	0.9855				

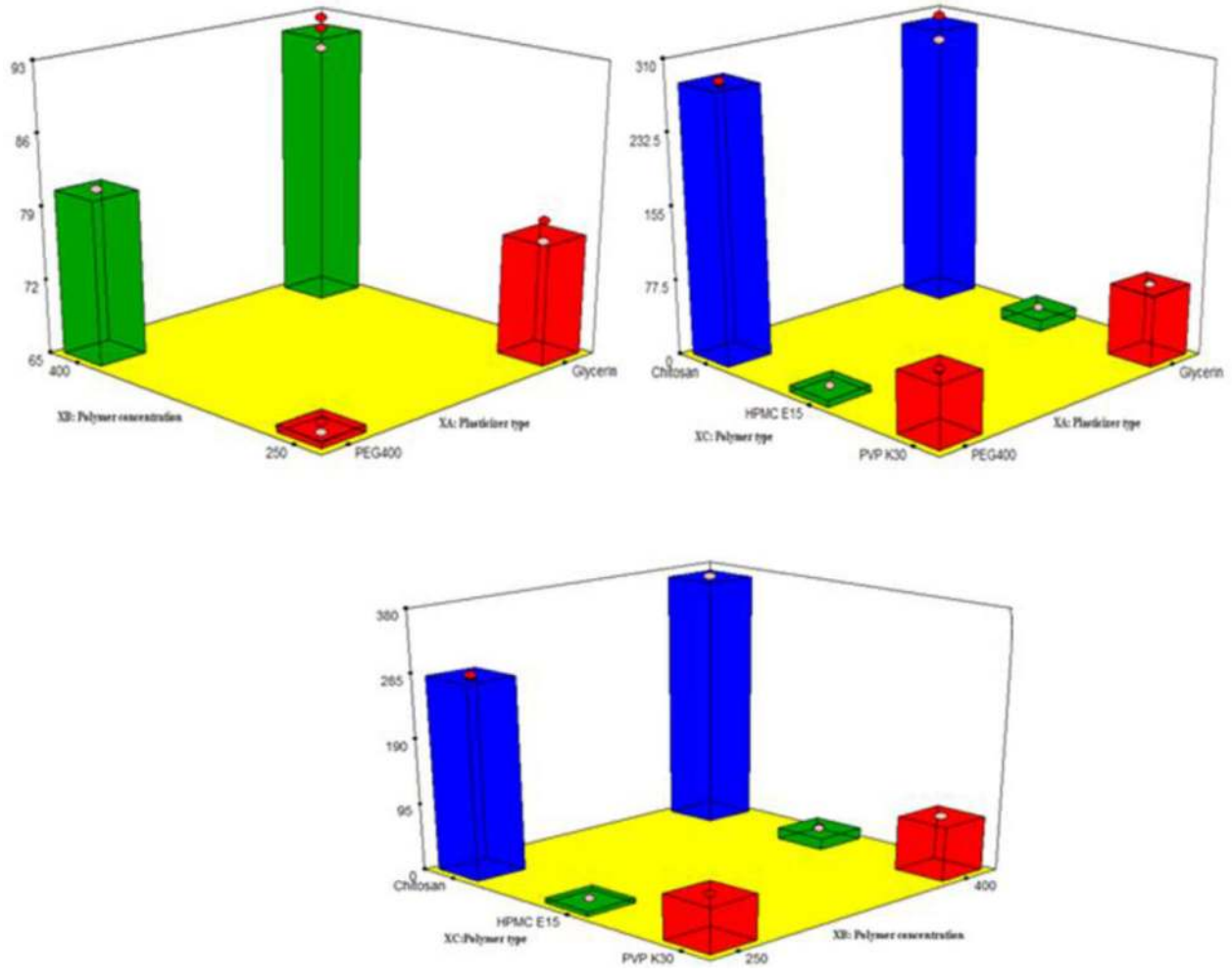

Figure 7 3-D surface plot for the combined effect of plasticizer type (X_A), polymer concentration (X_B) and polymer type (X_c) on (Y_1): DT. [Colour figure can be viewed at wileyonlinelibrary.com]

In-vitro drug dissolution test

Drug dissolution from the ODS involves water diffusion, relaxation of polymer chains, swelling and erosion of the strip.[42] Immediate and fast release of all strips formulae were observed at early time points. Amount of TML dissolved from different prepared ODSs was found to be ranged from 29% to 80% after 5 min as shown in Figures 5 and 6.

Statistical analysis. Statistical analysis of the full factorial ($3^1.2^2$) design batches was performed by ANOVA. To evaluate the contribution of each factor with different levels to the response, analysis of variance [classical sum-of-squares-Type II] ($P < 0.0001$) was performed using Design Expert 8.0 (STAT-EASE) software, enlisted in Table 4. The influence of each factor on the response and the response surface plots (graphically) were generated using the same software. The mathematical models developed for all the dependent variables using statistical analysis software are shown in Equations (1 and 2):

$$DT = +133.83 - 10.33 * X_A + 10.17 * X_B - 61.08 * X_C[1] - 119.25 * X_C[2] \quad (1)$$

$$\%Q_{5\,min} = +45.89 + 1.78 * X_A - 7.78 * X_B - 16.72 * X_C[1] + 27.28 * X_C[2] \quad (2)$$

For Y_1 as represented in Table 4 and Figure 7, changing plasticizer type (X_A) from PEG 400 to glycerine significantly affects DT ($P < 0.0001$). Using glycerine as a strip plasticizer showed a longer DT compared by using PEG400. A significant effect on DT ($P < 0.0001$) is

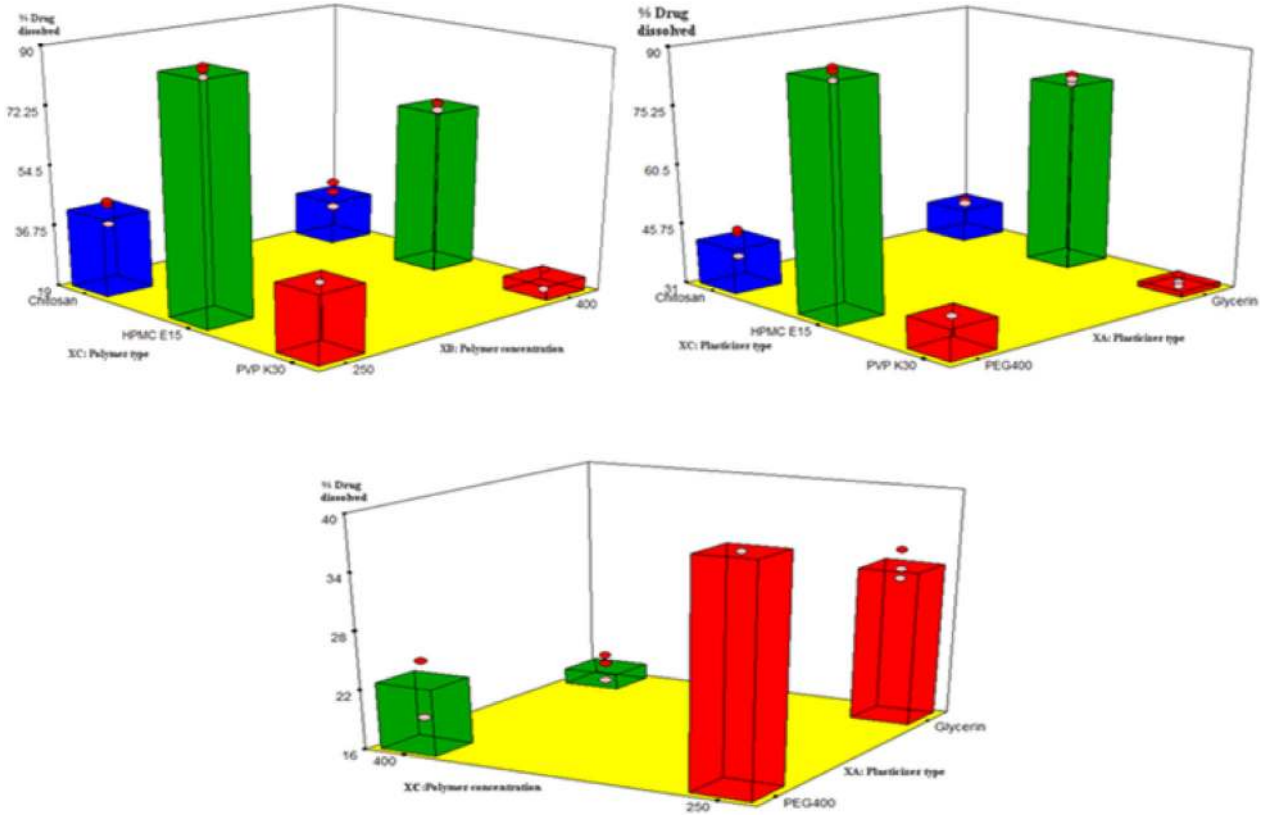

Figure 8 3-D surface plot for the combined effect of plasticizer type (X_A), polymer concentration (X_B) and polymer type (X_c) on (Y_2) $\%Q_{5min}$. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 5 Stability test parameters for optimized formula (F9)

Days	Appearance	Weight (mg)	Thickness (mm)	Folding endurance	Drug content (%)	Surface pH	DT (s)	$\%Q_{5min}$
60	Transparent	24.7 ± 0.4	24.1 ± 0.33	160 ± 0.52	99 ± 0.08	6.4 ± 0.75	9 ± 0.15	99 ± 2.3
120	Transparent	23.9 ± 0.27	23.4 ± 0.69	169 ± 0.79	97 ± 0.11	6.8 ± 0.98	7 ± 0.35	96 ± 1.6
180	Transparent	24.15 ± 0.9	24.2 ± 0.46	165 ± 0.36	95 ± 0.43	6.7 ± 0.23	10 ± 0.36	93 ± 1.4

observed by changing polymer concentration (X_B) from 250 to 400. DT of ODSs was found to be significantly increased by increasing polymer amount. Using PVP K30 or HPMC E15 or chitosan (X_c) affects the DT of ODSs of TML significantly ($P < 0.0001$), HPMC E15 showed the lowest DT among the prepared ODSs, and it was found that using HPMC in the strips caused a significant reduction in disintegration time which may be attributed to exhibiting greater hydrophilic character.[29]

Figure 8 and Table 4 of Y_2, using either PEG 400 or glycerine as a plasticizer (X_A), were found to significantly affect $\%Q_{5min}$. By using PEG 400 as a plasticizer, a higher per cent dissolved is generated when compared with glycerine. So, faster disintegration rate was suggested for prepared ODSs using PEG 400. Generally, water soluble plasticizers tend to leach out when exposed to dissolution medium and thereby create pores, which allow faster drug release. The increase in water soluble plasticizers concentration causes more pore formation resulting in a very fast release.[43] Changing polymer concentration (X_B) from 250 to 400 mg significantly affects the $\%Q_{5min}$ ($P < 0.0001$). It was found that the amount dissolved of TML was increased by using polymer in concentration of 250 mg when compared with using 400 mg polymer. It was reported that the increase in

Table 6 Effect of timolol orally dissolving strips on intraocular pressure in rabbits

	IOP (mmHg)			
	Groups			
Days	Normal	Betamethasone	TML eye drop	TML ODSs
1	9.40 ± 0.37	9.33 ± 0.43	9.42 ± 0.65	8.90 ± 0.48
2	9.83 ± 0.41	10.34 ± 0.36	11.12 ± 0.93	10.84 ± 0.68
3	8.90 ± 0.32	12.10 ± 0.40^{aaa}	11.57 ± 0.89^{aaa}	11.96 ± 0.74^{aaa}
4	10.20 ± 0.55	13.59 ± 0.67^{aaa}	14.08 ± 0.91^{aaa}	14.86 ± 0.70^{aaa}
5	9.20 ± 0.68	15.11 ± 0.74^{aaa}	16.33 ± 0.83^{aaa}	16.82 ± 1.02^{aaa}
6	8.95 ± 0.64	16.40 ± 0.78^{aaa}	17.15 ± 0.77^{aaa}	18.07 ± 0.83^{aaa}
7	9.58 ± 0.40	17.89 ± 0.91^{aaa}	18.14 ± 0.82^{aaa}	18.96 ± 0.82^{aaa}
8	10.47 ± 0.54	18.70 ± 1.04^{aaa}	14.78 ± 0.86^{aaa,bbb}	14.22 ± 0.46^{aaa,bbb}
9	9.15 ± 0.34	19.12 ± 0.88^{aaa}	13.27 ± 0.62^{aaa,bbb}	12.52 ± 0.41^{aaa,bbb}
10	9.40 ± 0.54	19.10 ± 0.83^{aaa}	12.86 ± 0.55^{aaa,bbb}	11.77 ± 0.79^{bbb}
11	9.85 ± 0.55	19.47 ± 0.97^{aaa}	12.13 ± 0.51^{aaa,bbb}	10.43 ± 0.61^{bbb,cc}
12	9.54 ± 0.59	19.43 ± 0.92^{aaa}	11.94 ± 0.57^{aaa,bbb}	9.59 ± 0.47^{bbb,ccc}
13	9.68 ± 0.65	19.65 ± 1.23^{aaa}	11.74 ± 0.43^{aaa,bbb}	9.48 ± 0.88^{bbb,ccc}
14	9.81 ± 0.52	19.77 ± 1.10^{aaa}	11.52 ± 0.46^{aaa,bbb}	8.50 ± 0.39^{bbb,ccc}

Values are represented as means ± SD (n = 6 animals). Significant difference compared with control group at $^{a}P < 0.05$, $^{aa}P < 0.01$, $^{aaa}P < 0.001$. Significant difference compared with betamethasone group at $^{b}P < 0.05$, $^{bb}P < 0.01$, $^{bbb}P < 0.001$. Significant difference compared with TML eye drops-treated group at $^{c}P < 0.05$, $^{cc}P < 0.01$, $^{ccc}P < 0.001$.

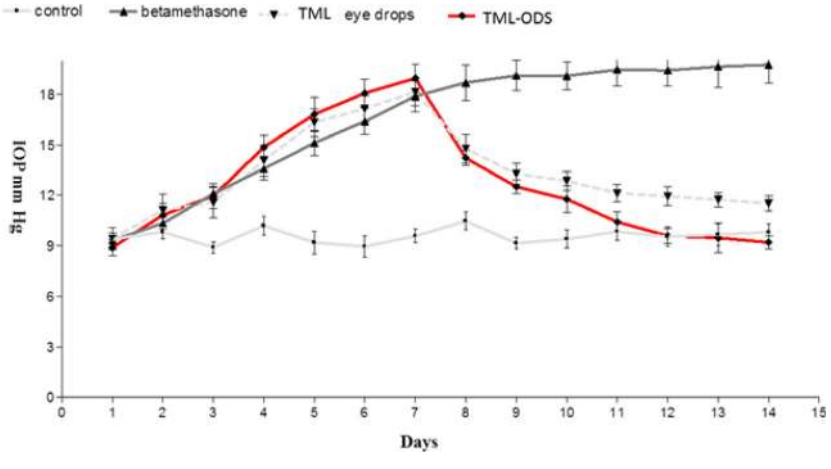

Figure 9 Dynamic changes of intraocular pressure of rabbits through the experiment period. [Colour figure can be viewed at wileyonlinelibrary.com]

polymer proportion will consequently increases the viscosity of gel layer and also results in gel layer with longer diffusional path length resulting in greater retardation of drug release.[44] Polymer type (X_c) was found to affect the per cent of drug dissolved significantly. Higher amount of TML was dissolved from ODSs based on HMPC E15, while slower TML dissolution rates were obtained from PVP K30 or chitosan ODSs. This may be attributed to the difference in the viscosity of utilized polymers. It was estimated that PVP K30 and chitosan have higher viscosity than HPMC E15. The obtained results showed Y_2 is inversely related topolymer viscosity.[45] According to the amount of TML dissolved from the strips, it could be arranged in a descending order as follows HPMC E-15 > PVP K30 > chitosan. HPMC E15 ODSs generated the highest amount of drug dissolved. The drug release mechanism from HPMC polymers involves water penetration and polymer relaxation to form a viscous rubbery region (gel layer), and this gel layer controls drug release by the viscous resistant force to drug diffusion or matrix erosion. Increase in HPMC concentration causes an increase in the viscosity and decreased drug release. The fast dissolution rate of TML from ODSs is attributed to the hydrophilicity of HPMC, which improves its interaction with the aqueous environment leading to erosion rate increasing. Also, cellulosic materials are linear polymers and not able to form virtual crosslinks at low concentrations.[46,47] Thus, the formed gels have low viscosity and the drug was quickly released.

Conclusively, from the polynomial equations of Y_1 and Y_2, there was a significant decrease in DT and significant increase in $\%Q_{5min}$; if PEG 400 used as a strip plasticizer, polymer concentration was 250 mg and HPMC E15 was used as strip forming polymer. According to ($3^1.2^2$) full factorial design, F9 met the required response (i.e. with desirability of 0.974), with minimized DT, and a high amount of drug dissolved after 5 min. So, it was selected for further assessment.

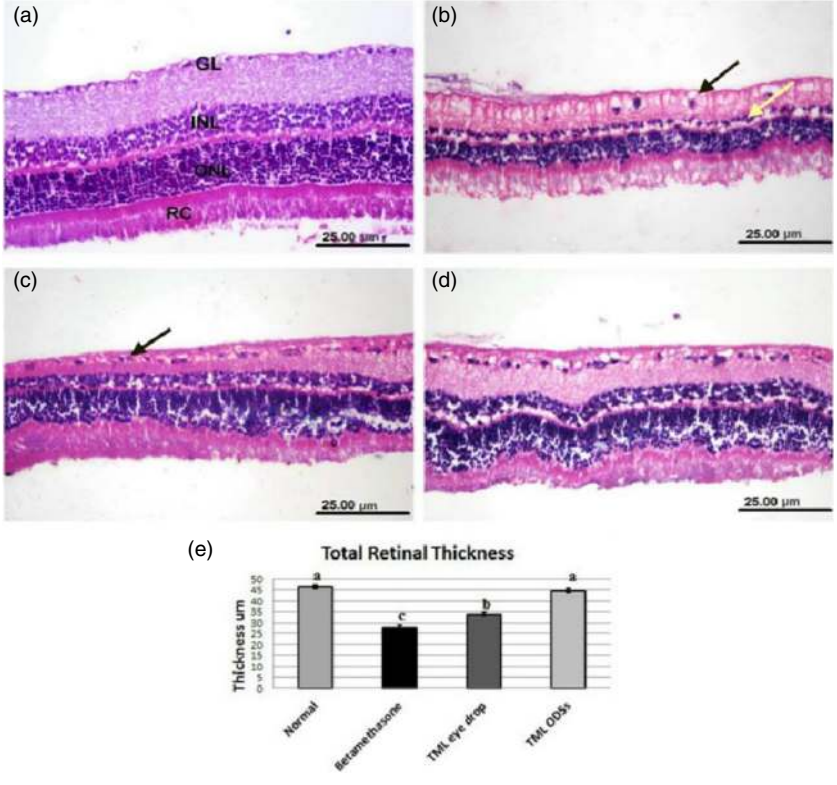

Figure 10 Micrograph of retinal histological H & E-stained sections (scale bar 25 µm); (a) negative control rabbit showing the normal histological structure of retinal layers, ganglionic cell layer, inner nuclear layer, outer nuclear layer and layer of rods and cones. (b) Glaucomic rabbit showing vacuolization of the ganglionic cells with pyknosis of their nuclei (black arrow) as well as thinning and atrophy of the inner nuclear layer (yellow arrow). (c) TML eye drops-treated rabbit showing pyknosis of some ganglionic cells (arrow). (d) TML ODS-treated rabbit showing normal histological structure and normal total retinal thickness. (e) The bar chart represents the total retinal thicknesses, data shown as mean ± standard deviation (n = 6); one-way analysis of variance was used for data analysis, and mean values with unlike superscript letters were significantly different (P < 0.05). [Colour figure can be viewed at wileyonlinelibrary.com]

Stability study

The results of stability study of optimized formula (F9) are shown in Table 5. The appearance of stored ODS remained unchanged. There was no significant change observed in weight of ODS, thickness, folding endurance, drug content, surface pH, DT and $\%Q_{5min}$ during 180 days.

In-vivo study and measurement of IOP

The results in Table 6 and Figure 9 revealed that subconjunctival treatment of rabbits with betamethasone significantly ($P \leq 0.05$) increased IOP starting from the third day after injection (13.59 ± 0.7). The IOP continued to be increased and reached its maximal level on the 9th day

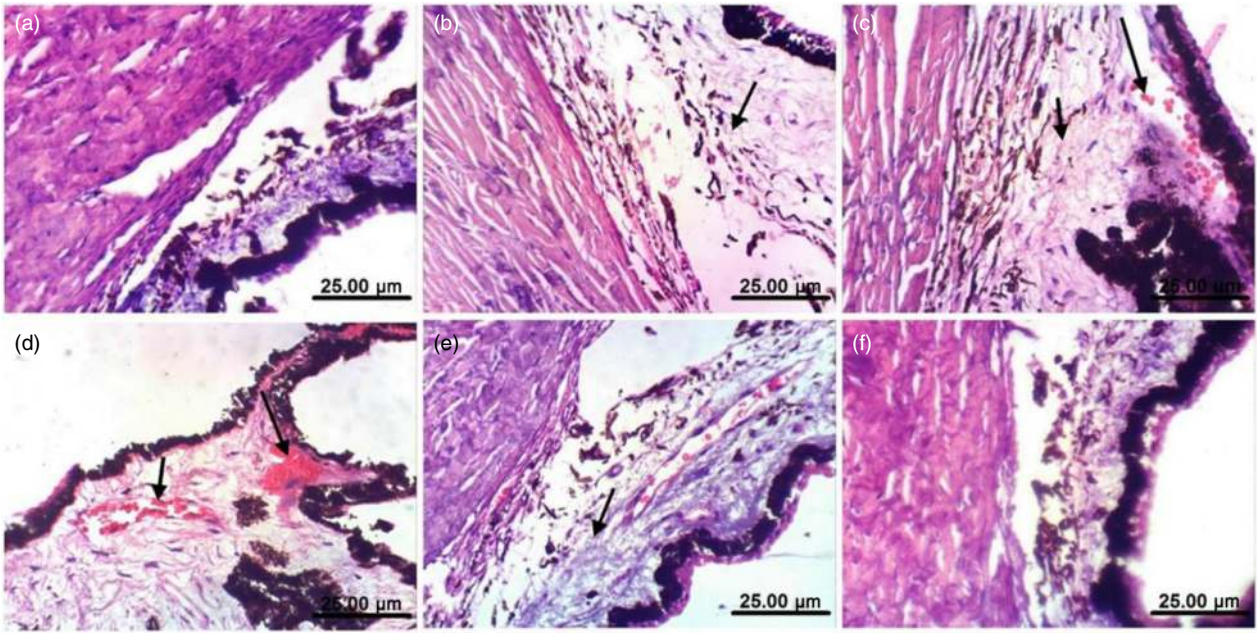

Figure 11 Micrograph of iridocorneal angle histological H & E-stained sections (scale bar 25 µm); (e of a) negative control rabbit showing no histopathological changes. (b–d) Glaucomic rabbit, (b) showing oedema (arrow), (c) showing oedema (short arrow) and focal haemorrhage (long arrow), and (d) congestion (short arrow) and haemorrhage (long arrow) in the ciliary processes. (e) Rabbit treated with TML eye drops showing slight oedema (arrow). (f) Rabbit treated with TML ODS showing no histopathological alterations. [Colour figure can be viewed at wileyonlinelibrary.com]

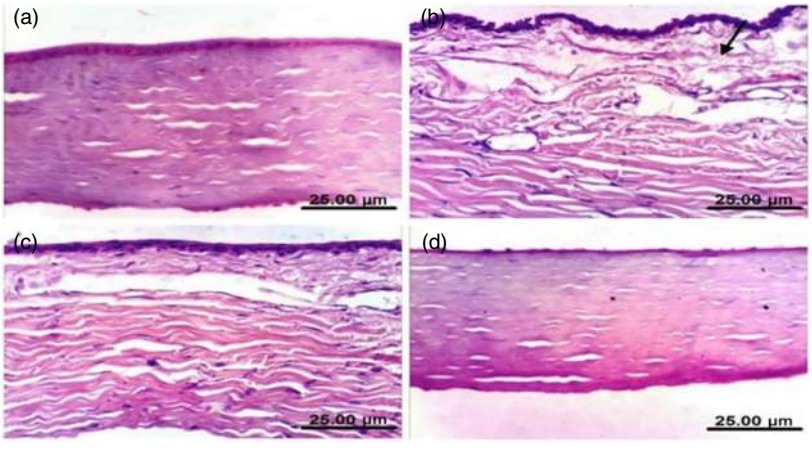

Figure 12 Micrograph of cornea histological H & E-stained sections (scale bar 25 µm); of (a) negative control rabbit showing no histopathological changes. (b) Glaucomic rabbit showing oedema in the corneal stroma (arrow), (c) rabbit treated with TML eye drops showing no histopathological changes. (d) Rabbit treated with TML ODS showing no histopathological alterations. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 7 Effect of timolol orally dissolving strips on glutathione and malondialdehyde content in experimentally induced IO hypertension in rabbits

Groups	MDA (nmol/ml)	GSH (mg/dl)
Control	8.54 ± 0.48	38.67 ± 1.50
Betamethasone	14.73^{aaa} ± 0.82	27.19^{aaa} ± 2.11
Timolol drops	14.22^{aaa} ± 1.12	$29.70^{aaa\ bbb}$ ± 1.94
TML ODSs	$11.86^{aaa\ bbbb\ ccc}$ ± 0.82	$36.12^{bbb\ ccc}$ ± 1.20

Values are represented as means ± SD (n = 6 animals). Significant difference compared with control group at $^{a}P < 0.05$, $^{aa}P < 0.01$, $^{aaa}P < 0.001$. Significant difference compared with betamethasone group at $^{b}P < 0.05$, $^{bb}P < 0.01$, $^{bbb}P < 0.001$. Significant difference compared with TML eye drops-treated group at $^{c}P < 0.05$, $^{cc}P < 0.01$, $^{ccc}P < 0.001$.

(19.12 ± 0.88) and approximately remained at this level until the end of the experiment. The control rabbits showed an IOP ranged between 8.95 ± 0.64 and 10.47 ± 0.57. Treating animals with TML drops or TML ODS resulted in significant ($P \leq 0.01$) reduction in IOP compared with corresponding betamethasone-treated rabbits starting from the 8th day until the end of experiment. The potent inhibitory effect of TML ODS on IOP compared with TML eye drops appeared at the 11th day of the treatment ($P \leq 0.01$). This inhibitory effect was continued until the 14th day. It is important to note that resultant decrease in IOP induced by TML ODS was non-significant compared with control rabbits staring from the 10th day until the 14th day. The in-vivo study revealed that TML formulated as ODSs decreased IOP significantly compared with TML eye drops. The effect of the TML ODSs appeared after 24 h of drug administration and then completed its inhibitory effect on IOP to be nearly comparable to IOP readings obtained from control rabbits.

Histopathological examination and histomorphometric analysis. Microscopically, retina of negative control rabbits revealed the normal histological structure of retinal layers, which composed from ganglionic cell layer (GL), inner nuclear layer (INL), outer nuclear layer (ONL) and layer of rods and cones (RC; Figure 10a). On the contrary, retina of glaucomic rabbits revealed variable histological alternations including necrosis of the ganglionic cells, vacuolization of ganglionic cells with pyknosis of their nuclei, loss of ganglionic cell layer, vacuolization of the inner and outer nuclear cell layers and thinning and atrophy of the inner and outer nuclear layers which was prominent in the inner nuclear layer (Figure 10b). Meanwhile, retina of rabbits treated with TML eye drops revealed improved histological picture, and the examined sections moderately restored total retinal thickness with pyknosis of some ganglionic cells (Figure 10c) and slight vacuolization of the inner nuclear cell layer. On the other hand, the histopathological lesions were markedly regressed in the retina of rabbits treated with TML ODSs, and the retina restored the normal histological structure and recorded no histopathological alterations and normal total retinal thickness (Figure 10d). Analyses of the total retinal thicknesses (measured from ganglionic cell layer to the layer of rods and cones) are illustrated in Figure 10e, data shown as mean ± SE. Furthermore, significant thinning in the total retinal thickness was recorded in glaucomic rabbits. There was significant decrease in the total retinal thickness (means 27.80 ± 0.32) when compared with control rabbits (means 46.38 ± 0.80). Moreover, there was moderate significant thinning (means 33.68 ± 0.51) in the group treated with TML eye drops. On the other hand, there was no difference in the

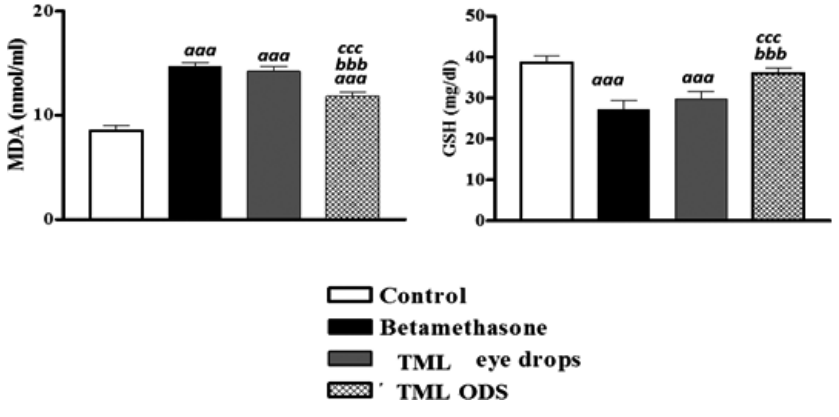

Figure 13 Effect of timolol orally dissolving strips on glutathione and malondialdehyde contents in experimentally induced intraocular pressure in rabbits. Values are represented as means ± SD (n = 6 animals). Significant difference compared with control group at $^{a}P < 0.05$, $^{aa}P < 0.01$, $^{aaa}P < 0.001$. Significant difference compared with betamethasone group at $^{b}P < 0.05$, $^{bb}P < 0.01$, $^{bbb}P < 0.001$. Significant difference compared with timolol eye drops-treated group at $^{c}P < 0.05$, $^{cc}P < 0.01$, $^{ccc}P < 0.001$.

retinal thickness of eyes (means 44.49 ± 0.90) from group treated with TML ODSs when compared with the control group. Moreover, histopathological examination of iridocorneal angle and cornea of control rabbits revealed no histopathological changes (Figures 11a and 12a). On the contrary, iridocorneal angle of glaucomatous rabbits revealed variable histopathological alternations summarized as oedema (Figure 11b and 11c), few inflammatory cells infiltration and focal haemorrhage (Figure 11c). Congestion and haemorrhage in the ciliary processes (Figure 11d) as well as oedema in the corneal stroma (Figure 12b) were also recorded in examined sections. Meanwhile, slight oedema was the only histopathological finding in iridocorneal junction in rabbits treated with TML eye drops (Figure 11e), associated with no histopathological changes in their cornea (Figure 12c). Markedly, improved picture was noticed in rabbits treated with TML ODSs; iridocorneal angle and cornea from those treated rabbits revealed no histopathological alterations (Figures 11f and 12d). Our results showed that TML ODSs formula is a potent antiglaucomic in addition to neuroprotective activity. The histopathological lesions observed in glaucomic rabbits such as ganglionic cells necrosis, vacuolar degeneration of nuclear cell layers and thin and atrophied retina were markedly regressed in the retina of TML ODSs treated rabbits.

The retina restored the normal histological structure and normal retinal thickness. Moreover, the iridocorneal junction in rabbits treated with TML ODSs revealed normal histopathological structure with no inflammation or oedema in the corneal layer. The neuroprotective effect of TML is controversial. It has been reported that TML displays a direct neuroprotective effect in experimental models of retinal injury which is in part due to an independent mechanism of its ocular hypotensive efficacy.[48] Moreover, other studies support a potential neuroprotective effect of β-adrenoceptor antagonists[49] although to a lesser extent than other molecules. On the other hand, Gross and his colleagues reported a negative action of TML as neuroprotective agent.[50]

Assay of oxidative stress markers. For further investigation of the neuroprotective effect of TML ODSs, we assessed the antioxidant activity of this formula. It has been reported that glaucoma patients had a lower level of antioxidant molecules such as glutathione (GSH). Dampening levels of antioxidant molecules may augment the optic nerve damage in those patients.[51] Our study showed that new TML ODS formula has a potent activity against oxidative stress compared with betamethasone and even to TML eye drops. These results were inconsistent with Refs. [52,53] who illustrated an antioxidant effect of TML in both corneal and endothelial tissues. This study revealed that betamethasone induced significant ($P < 0.001$) increase in MDA blood level as well as significant ($P < 0.001$) decrease in GSH compared with control rabbits. On the other hand, both TML eye drop and TML ODSs significantly ($P < 0.001$) inhibited MDA level and augmented GSH activity compared with betamethasone-treated rabbits. It is to be noted that rabbits treated with TML ODSs showed significant antioxidant activity represented as decreased MDA level and increased GSH activity compared with TML drops-treated rabbits (Table 7, Figure 13).

Conclusion

F9 exhibited flash release in remarkable disintegration time. The optimized TML ODS formula decreased IOP significantly compared with TML eye drops. In conclusion, this study suggests a potential alternative for TML eye drops by using faster drug release and easier administration accompanied by persistent patient compliance for enhanced therapeutic activity for POAG patients, who also may be accompanied by hypertension.

Declarations

Conflict of interest

The Authors declare that they have no conflicts of interest to disclose.

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Author contributions

Yasmin El-Feky, Dalia Mostafa and Dalia El-Telbany designed the study. Sherin Zakaria, Dalia El-Telbany, Yasmin El-Feky, Kawkab Ahmed, Ahmed Fayez and Dalia Mostafa performed experiments. Yasmin El-Feky and Majid Al-Sawahli performed statistical analysis and drafted manuscript. Ebtesam Alolayan, Majid Al-Sawahli and Rania El-Telbany contributed to the interpretation of the results. All authors discussed the results and contributed to the final manuscript.

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