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Ophthalmic delivery of sparfloxacin from in situ gel formulation for treatment of experimentally induced bacterial keratitis

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The objective of the present work was (1) to develop an *in situ* gelling ophthalmic delivery system by combining pluronic F127 and pluronic F68, with sparfloxacin; and (2) to examine the influence of incorporating a mucoadhesive polysaccharide such as sodium hyaluronate on the healing property due to bacterial keratitis. The formulations (F1–F6) were sterilized by gamma irradiated using Co^{60}. Ultraviolet (UV) and infrared (IR) spectra studies were performed on sterilized and non-sterilized formulae. The formulations were evaluated for rheological characteristics, *in vitro* release behavior, and efficacy against induced bacterial conjunctivitis in rats' eyes. Moreover, histopathological evaluations were also done. All the samples passed sterility tests, and no change in physical appearance of the formulae due to gamma radiation was observed. The IR spectra of the formulae before and after sterilization showed similar peaks which confirmed that no ingredient was affected by gamma radiation. The formulations showed a flow index of 0.116–0.493 indicating pseudoplastic flow behavior. The release behavior of all formulae was non-Fickian anomalous release. The different formulae used to overcome the pathological alterations, produced by bacteria infections varied among each other depending on the duration of treatment; however, the effectiveness of formulation was arranged as F5, F4 and F3, respectively. The developed formulations were therapeutically efficacious, and provided sustained release of the drug over a 24-hour period. A better improvement in artificially induced bacterial conjunctivitis in rats' cornea was observed with the developed formulae; thus it can be considered as a viable alternative to conventional eye drops.

Keywords: *in situ* gel; bacterial keratitis; Sparfloxacin; IR; rheology

Introduction

Controlled and sustained drug delivery systems have recently become standard in modern pharmaceutical design. Intensive research has been undertaken in achieving much better drug product effectiveness, reliability, and safety.[1,2] The eye presents a challenge in the development of sustained or controlled release systems due to its sensitivity and effective protection mechanisms, such as lachrymal secretion and blinking reflex, which cause rapid drainage of bioactive agents after topical administration.[3] The normal drainage of an instilled drug dose commences immediately upon instillation and is essentially completed within 5 min.[4] The short precorneal residence time results in low bioavailability and frequent dosing is usually needed to compensate for the decreased precorneal drug concentration.[5] To increase ocular bioavailability and duration of the drug action, various ophthalmic vehicles, such as viscous solutions, ointments, gels, or polymeric inserts, have been used.[6] Increasing viscosity by incorporating polymers into aqueous vehicles seems a possible way to slow down drug elimination from the conjunctival sac of the eye.[7–9] *In situ* gels are conveniently dropped as a solution into the conjunctival sac, where they undergo a transition into a gel with its favorable residence time. The sol-gel transition occurs as a result of a chemical/physical change induced by the physiological environment. This type of gel combines the advantage of a solution being convenient for the patient with the favorable residence time of a gel for enhancing ocular bioavailability. Practically, the sol-gel transition can be induced by a shift in the pHas for cellulose acetate phthalate; a shift in temperature as for the thermogelling poloxamer 407;[10–13] or by the presence of cations as for deacetylated gellan gum and alginates. Thus, with the use of these *in situ* gelling systems, the residence time of the drug in the eye is increased, reduced dose concentrations and dosing frequency, and improved patient acceptability. Drug delivery systems based on the concept of *in situ* gel formation should provide these properties and most of them are concerned with ophthalmic disease treatment.[14,15]

One of the thermoreversible polymer analogues is poloxamer 407 (Pluronic- F127). It consists of hydrophilic polyoxyethylene units (70%) and hydrophopic polyoxypropylene blocks (30%).[16] At a concentration of 15% or higher in aqueous solution, PF127 is transformed from a low viscosity solution to a semisolid gel upon heating from 4 °C to a temperature greater than 23 °C; this thermogelation is reversible upon cooling.

Sparfloxacin, a third-generation fluoroquinolone derivative,[17–19] is a potent antibacterial agent active against a wide range of Gram-positive and Gram-negative organisms including Streptococcus pneumonia, Staphylococcus aureus. It is structurally 5-amino-1-cyclopropyl-7-(cis-3,5-dimethyl)-1-piperozinyl)-6,8-difluoro-1,4-dihydro-4-oxo-3- quinoline.

Hyaluronic acid (HA), also known as sodium hyaluronate or hyaluronan, is an endogenous high molecular weight linear

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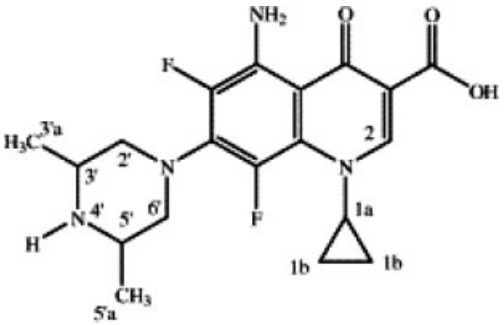
polysaccharide of a repeating disaccharide unit that has a number of embryologic and wound-healing properties, including the facilitation of cell migration and differentiation during tissue formation and repair.[20–25] Sodium hyaluronate is a biocompatible mucoadhesive polysaccharide with a repeating unit of ß-D glucuronic acid and N-acetylglucosamine, which has been proven to increase the tear film stability and reduce subjective ocular discomforts.[26,27]

The objective of the present work was to develop *in situ* gelling ophthalmic delivery system with a suitable phase transition by combining pluronic analogue namely pluronic-F127 (PF127) and pluronic-F68 (PF68), with sparfloxacin, used in the treatment of external eye infections. Also, to examine the influence of incorporating mucoadhesive polysaccharide, sodium hyaluronate (HA-Na) in different concentrations on the healing property in case of experimentally induced bacterial keratitis.



Materials and Methods

Materials

Sparfloxacin was obtained as a gift sample from Global-Nabi Co. (Cairo, Egypt) Pluronic-F127(PF127), Pluronic F68, and benzalkonium chloride was purchased from Sigma Chemical Co. (St Louis, MO, USA). Carboxymethylcellulose sodium (CMC) was purchased from BDH Chemicals Ltd (Poole, England). Hyaluronic acid sodium salt from streptococcus equi sp. was purchased from Sigma-Aldrich ChemieGmbh (Steinheim, Germany). Cellophane dialysis membrane (M.W cut off 3600) (Fisher Scientific, Ltd, Loughborough, Leicestershire, UK). Fluorescein Eye Test Strips (Odyssey Medical Inc., US). Hematoxylin, Fluka AG, Buchs SG Switzerland Eosin, from BDH Chemicals Ltd; The Britch Drug House (Poole, England). Fisher Scientific (H&E). Formaline (Adwic). *Staphylococcus aureus* subsp. aureus ATCC® 29737™ and *Escherichia coli* ATCC® 10536™ were supplied by LyfoCults® Plus, PML Microbiologicals, Inc., BioMerieux, Inc. (Wilsonville, OR, USA), were used in induction of corneal ulcer.

The composition of simulated tear fluid (STF) used was sodium chloride 0.670 gm; sodium bicarbonate 0.200 gm; calcium chloride $2H_2O$ 0.008 gm; and purified water 100 gm.

Culture media and micro-organism

Broth thioglycollate culture was intended for the growth of aerobic and anaerobic bacteria, while broth sabarout culture was used for the growth of fungi.

Animals

The animals used for *in vivo* experiments were 24 adult young male white albino rats weighing 150–200 gm, from the Department of Central Animal House (NODCAR). This study was conducted in accordance with ethical procedures and policies approved by Animal Care and Use Committee of National Organization for Drug Control and Research (NODCAR), Egypt following the 18th WMA General Assembly, Helsinki, June 1964 and updated by the 59th WMA General Assembly, Seoul, October 2008.

Equipment

Cone and plate viscometer, model DV-III Brookfield Engineering Lab. Inc. (Middleboro, MA, USA) with spindle 52. Hot plate with magnetic stirrer, MSH-420, BOECO (Hamburg,Germany). UV-Spectrophotometer (Shimadzu, Kyoto, Japan). Electronic analytical balance, JT3003, Shimadzu, Japan. Electronic analytical balance, JT3003 (Shimadzu, Kyoto, Japan). Microtome (leica RM2 125) Richmond, IL, (USA). Diffusion cell for *in vitro* release. FT-IR-84005 (Shimadzu, Kyoto, Japan). pH 211 microprocessor-based Bench pH/mV/°C Meter, HANNA Instruments (Sanneola di Rubano-PD Italy). OPTICH imaging system (Heidelberg Engineering, Dossenheim Germany): It consists of light microscope equipped with a colored digital camera, which is linked to a computer via an image-capturing board, where the *Image-Pro Plus* software package was installed A Mediacybernetics, Inc., Georgia Avenue, Suite, Silver Spring, MD, USA. (Version 5.0.1, for Windows 2000 and XP Professional). Slit Lamp Biomicroscope, Topcon, (Tokyo,Japan). Gamma irradiation using Co^{60} as irradiation source.

Preparation of PF127 sparfloxacin sol-gel formulations

The sol-gels were made on a weight basis using the modified cold method.[28] PF127 was mixed with the drug, isotonic agent, and water, refrigerated at 4 °C, and stirred periodically until a homogeneous solution was obtained. Viscosity-enhancing agent was also prepared and 3% CMC was used. To study the effect of hyaluronate on the rheological release properties, and its

Table 1. Composition of in-situ geling formulations of sparfloxacin

Ingredients	F1	F2	F3	F4	F5	F6
Sparfloxacin (% w/v)	0.3	0.3	0.3	0.3	0.3	0.3
Pluronic F127 (% w/v)	21	15	15	15	15	15
Pluronic F68 (% w/v)	0	10	10	10	10	10
CMC (% w/v)	0	0	0	0	0	3
sodium hyaluronate (% w/v)	0	0	0.1	0.3	0.5	0
Benzalkonium chloride (% w/v)	0.05	0.05	0.05	0.05	0.05	0.05
Phosphate buffer pH 6.8 (ml) Q.S to	100	100	100	100	100	100

effectiveness on bacterial keratitis, different concentrations of HA were used. Briefly, the drug, and mucoadhesive polymers were stirred in the calculated amount of distilled water at room temperature. The dispersions were cooled down to 4 °C; the poloxamer was added slowly with continuous stirring (thermostatically controlled magnetic stirrer). The dispersions were then stored in a refrigerator until clear solutions were obtained.[29] Table 1 shows the composition of all the formulations (F1–F6). The pH of all formulations was measured and it was found to be in the range of 6.7–7.4. Afterwards, the solutions were stored in vials (glass type I) and sterilized by autoclaving and or gamma radiation.

Evaluation of the formulations

The developed formulations were evaluated for drug content by ultraviolet (UV) spectrophotometry at 290 nm, pH measurements, and sterility Table (2).

Sterilization by gamma irradiation and sterility test

According to the US Pharmacopeia, all ophthalmic preparations should be sterile; therefore, the test for sterility is a very important evaluation parameter. All exposed samples were gamma irradiated using Co^{60} as irradiation sources at 20 °C, 1013.25mBar at the National Institute for Standards/Ionzing Radiation Metrology Lab (IRML). The dose rate of 1.25 mGy/min at 100 cm was measured using Secondary Standard Dosimetry System (Unidose 10001 Serial No. 10522 and its ionization chamber, PTW 30013, Serial No. 2016) with a combined uncertainty $>\pm 1.75\%$. The sterility test was applied on the prepared formulae (F1–F5) after sterilization by gammairradion to be sure that there was no contaminating micro-organisms. It was carried out under specific conditions using direct inoculation of the culture media with the product to be examined.[30]

UV scanning

UV scanning was done to investigate any interaction between the drug and polymers as well as to study the effect of gamma radiation on sparfloxacin. The sterilized and non-sterilized viscous systems were dissolved in STF of pH 7.4. The solution was filtered through Whatman filter paper No.4, and was scanned for UV absorption between 200 and 400 nm. UV scans of the plain formulations were also run and compared with the medicated formulations.

IR spectroscopy studies

Interaction studies were carried out by IR to prove any interaction between the drug and polymers as well as to study the effect of gamma radiation on saprfloxacin. The IR spectra studies were performed on sterilized and non-sterilized viscous systems and the spectra were compared with the IR spectra of the pure drug and plain formulations.

Rheological characterization

The viscosity determinations of prepared formulations (F1–F6) were carried out at 25 °C using Brookfield DV-111 viscometer with spindle 52 equipped with thermostat bath. The prepared solution was allowed to gel in the STF and then viscosity was measured. To obtain the ascendant curve, the angular velocity was increased gradually from 0.1 to 250 rpm and then, the angular velocity was reversed with gradually decreasing speeds (250–0.1 rpm) to obtain the descendant segment. The average of the two readings was used to calculate the viscosity. The consistency index and flow index were calculated from the Powerlaw equation:

$$\tau = K r^n \quad (1)$$

Where: τ is shear stress; r is shear rate; K is consistency index; and n is flow index.

Taking log of both sides,

$$\text{Log } \tau = \log K + n \log r \quad (2)$$

Thus, from the plot of log of shear stress *v/s* log of shear rate, the slope of the plot representing flow index and antilog of the y-intercept indicating consistency index were calculated.The two dimensionless quantities – the consistency index (k) and the flow index (n) – characteristic for each formulation were obtained. If $n = 1$, this indicates Newtonian behavior while if (n) is less than 1, this corresponds to shear thinning flow. The lower the value of (n) the more shear thinning the formulation.[31–33] Consistency index is a measure of consistency and is equivalent to apparent viscosity at a shear rate of 1 Sec^{-1}.The flow index *n* is a measure of the deviation of a system from Newtonian behaviour ($n = 1$). A value of $n < 1$ indicates pseudoplastic flow or shear thinning; $n > 1$ indicates dilatant or shear thickening flow. Flow index confers an idea of the flowability of the formulation from the container. Generally, the thicker the base, the lower the flow index.

In vitro release study

The *in vitro* release of sparfloxacin from sol-to-gel systems (F1–F6) was carried out through cellophane membrane using modified apparatus. Studies were also carried out for the marketed ciprofloxacin eye drops in order to compare the release profile with the prepared *in situ* gelling system of sparfloxacin. The dissolution medium used was freshly prepared STF pH 7.4. Cellophane membrane, previously soaked overnight in the dissolution medium, was tied to one end of a specifically designed glass cylinder (tube open at both ends). 1 ml of formulation (equivalent to 3 mg of sparfloxacin) was accurately placed into this assembly. The cylinder was attached to a stand and suspended in 100 ml of dissolution medium maintained at 34 ± 1 °C; the membrane was just touching the receptor medium surface. The dissolution medium was stirred at 50 rpm using a magnetic stirrer. Aliquots, each of 5 ml volume, were withdrawn from the release medium at time intervals 0.25, 0.5, 0.75,1.0, 1.5, 2, 4, 6, 8,10,12, and 24 h and each aliquot was replaced by equivalent amount of fresh STF. The samples were filtered through Whatman filter paper No. 4 and suitably diluted with the dissolution medium and analyzed by UV-Spectrophotometry at 290 nm.[17] The concentration of drug was determined from a previously constructed calibration curve. The release experiments were run in duplicates and the results were averaged.

Analysis of drug release data

The data obtained from the *in vitro* release experiments were analyzed by the following commonly used exponential equation[34]:

$$Mt/M = Kt^n$$
$$\text{Log } Mt/M = \log k + n \log t \quad (3)$$

Where Mt/Mis the fraction of released drug at time t; k is the release constant and is dependant on structural and geometric

characteristics of the drug/polymer system; and n is the release exponent indicative of the release mechanism. When n = 0.5, the drug is released from the polymer with a Fickian diffusion mechanism (Higuchi model). If 0.5 < n < 1, this indicates anomalous or non-Fickian release, while if n = 1, this indicates zero-order release.[34]

In vivo and histopathological study of the eye cornea

This study was conducted in accordance with ethical procedures and policies approved by Animal Care and Use Committee of the National Organization for Drug Control and Research (NODCAR), Egypt. Twenty-four adult albino rats with apparently healthy eyes were used in this study. The animals had free access to water and a standard rodent chow prior to and during the whole period of study. All animals were completely healthy with no history of ocular injury. They were housed in a temperature-controlled room and ventilated cages.

Induction of corneal ulcers by bacterial infection

A corneal ulcer, or ulcerative keratitis, or eyesore is an inflammatory or more seriously, infective condition of the cornea involving disruption of its epithelial layer with involvement of the corneal stroma.

Procedures

All rats except those of the first group (normal control) were sedated and anesthetized with intramuscular injection of ketamine hydrochloride (60 mg/kg) and the two eyes of each animal were injected with bacteria. To produce keratitis, 10 colony-forming units (0.1 mL) of clinical bacterial strains of *Staphylococcus aureus* and *Escherichia coli* in logarithmic growth phase were injected into the corneal stroma in the two eyes of rats.[35,36] The injection was performed with a 30-gauge needle into the stroma in the central part of the cornea. Twelve hours after the injection, the infected rats were divided into 8 groups of 3 rats each, and 50 µL of the different preparations was dropped into the conjunctival sac of each rat from group 3 to group 8. After dosing, the lids were held together for a few seconds in order to avoid loss of the dosage form. During rheology and *in vitro* release study of formula (F6) that contained 3% CMC, instead of forming *in situ* gel it became firm gel, so it was excluded in *in vivo* study. The other formulae of sterilized sol-to-gel formulae (F1–F5) were administered every 24 h, while the marketed eye drops were instilled 3 times daily.

Group (1): normal rat, non-infected, non-treated served as normal control.
Group (2): Infected rats, non-treated served as positive control.
Group (3): Infected rats treated with marketed eye drops, three times daily and kept as reference standard.
Group (4): Infected rats whose eyes were exposed to the application of one drop of the formula F1every 24 h.
Group (5): Infected rats whose eyes were exposed to the application of one drop of the formula F2 every 24 h.
Group (6): Infected rats whose eyes were exposed to the application of one drop of the formula F3 every 24 h.
Group (7): Infected rats whose eyes were exposed to the application of one drop of the formula F4 every 24 h.
Group (8): Infected rats whose eyes were exposed to the application of one drop of the formula F5 every 24 h.

Diagnosis of corneal ulcers

Diagnosis is done by direct observation under magnified view of slit lamp revealing the ulcer on the cornea. The use of fluorescein strip, which is taken up by exposed corneal stroma and appears green, helps in defining the margins of the corneal ulcer, and can reveal additional details of the surrounding epithelium. Practically, when fluorescein paper is applied to the eye, the ulcer will absorb the dye and the orange color of fluorescein paper changes to a green color, making diagnosis easy.

Corneal histopathological studies

Both positive control (group 2) and one rat of each group were sacrificed 24 h after bacterial inoculation to prove the presence of ulcer in contradistinction to the corneal section of group 1 (normal control) that showed healthy intact cornea. To investigate the effect of the prepared formulae on the infected animals, the eyes of rats from group (3) to group (8) were observed daily along the whole period of the study for signs of clinical cure; fluorescein paper test was done to prove ulcer healing. The healed ulcers were detected by no change of the orange color of fluorescein paper by slit lamp. The rats were subjected to histopathological evaluation and photomicrographs were taken by light microscope. The rats were anesthetized by ether inhalation, and their eyes were enucleated carefully. The corneas were excised along the limbus with scissors of both infected and non-infected rats and specimens of the cornea were taken. They were fixed in 10% neutral buffered formalin and were processed for light microscopy to prepare 5 µ thick paraffin sections and stained with Hematoxylin and Eosin (H&E). Specimens were examined and photographed under light microscope equipped with a colored digital camera, which is linked to a computer via an image-capturing board.[37,38]

Results and Discussion

Sterilization by gamma irradiation and sterility test

According to the US Pharmacopeia method, satisfactory results indicate that no contaminating micro-organisms were found in the sample examined. All the samples passed the sterility test, and no change in physical appearance of the prepared formulae due to gamma radiation was observed.

UV scanning

The UV absorption spectra for pure drug formulations before and after sterilization were quantitatively similar, with similar λ max at 290 nm. When assayed, almost 97.5% of the loaded drug was recovered from the gel formulations.

IR spectroscopy studies

The IR spectra of the formulations before and after sterilization showed similar peaks of sparfloxacin (Figure 1A) at 1700 cm^{-1} due to the C═O stretching vibration and a doublet between 3300 and 3450 cm due to the N-H stretching vibration, which confirmed that no ingredient was affected by gamma radiation in the dosage form and no irradiated product was formed within the dosage form. The main peaks of the dosage form were similar to those of the pure drug in the IR spectra. The IR spectra of plain formulation before and after sterilization exhibited similar patterns, which indicated that there was no effect of gamma radiation on the ingredients of the formulations as shown in Figures 1B and IC.

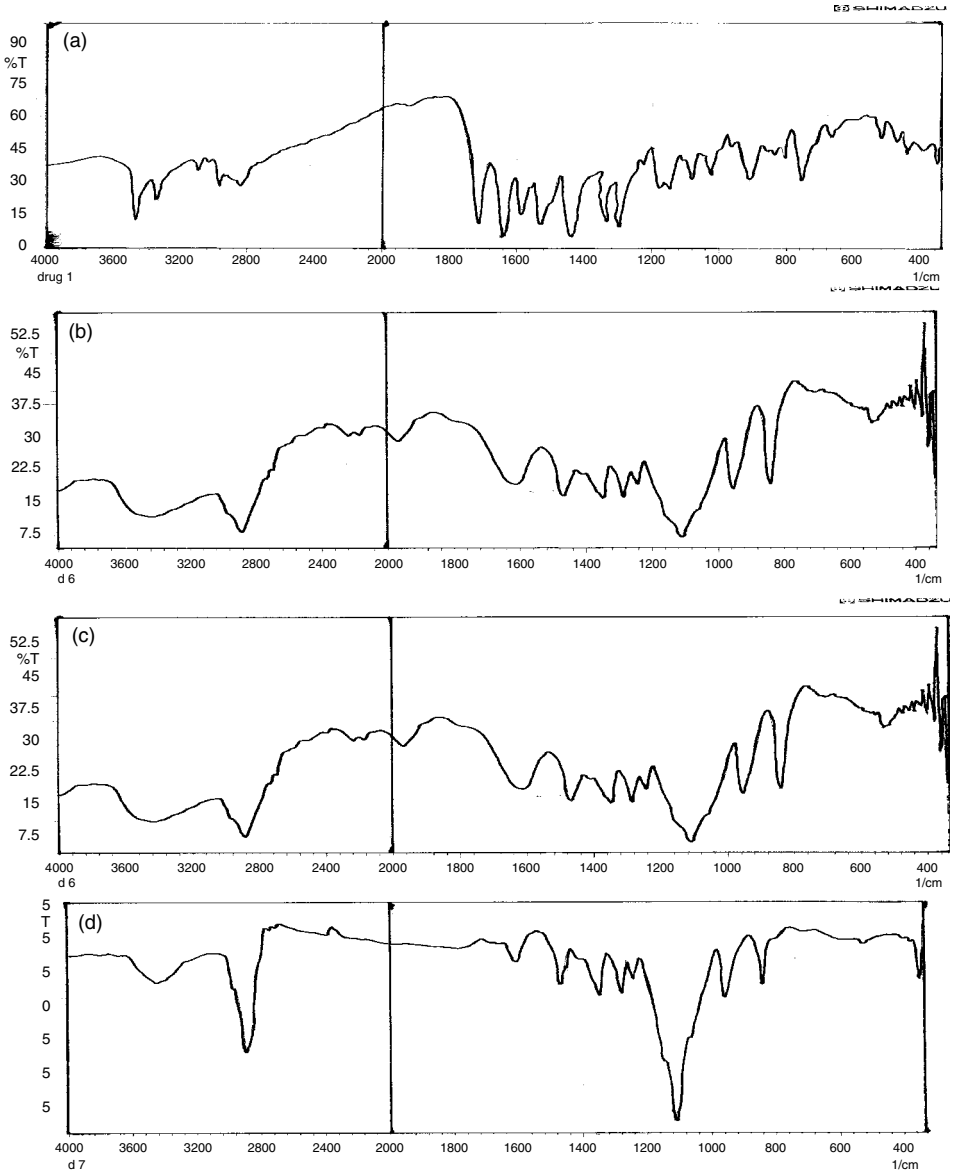


Figure 1. Infrared spectra of (a) drug, (b) plain sol -gel,(c) drug in sol- gel. before sterilization (d) drug in sol- gel. after sterilization.

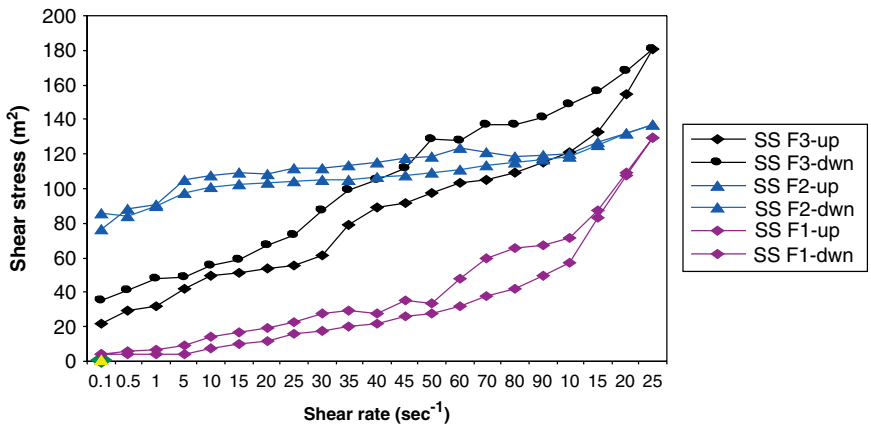


Figure 2. Rheograms of in situ gel formulations (F1-F3).

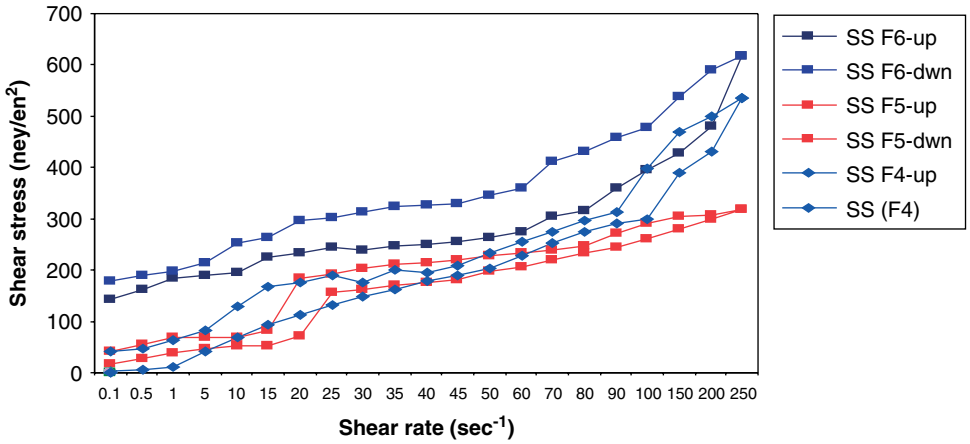


Figure 3. Rheograms of in situ gel formulations (F4-F6).

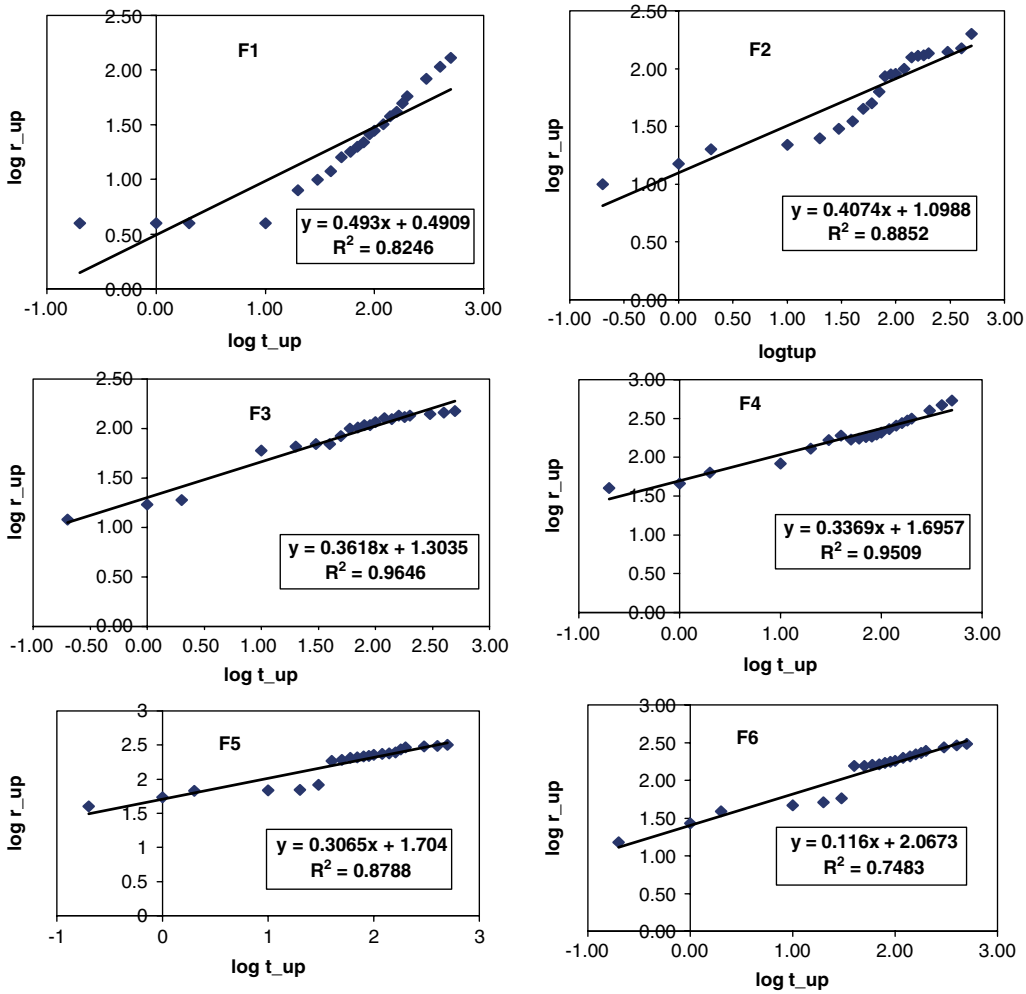


Figure 4. Fitting to the power law model of F1-F6, the slope of the plot representing flow index and antilog of the y-intercept indicating consistency index.

Rheological characterization

The formulations were shear thinning and an increase in shear stress was observed with an increase in angular velocity (pseudoplastic rheology; Figures 2 and 3). The administration of ophthalmic preparations should influence the pseudoplastic character of the precorneal tear film as little as possible.[39] Since the ocular shear rate is very large, ranging from 0.03 S^{-1} during interblinking periods to 4250–28500 S^{-1} during blinking, viscoelastic fluids with a viscosity that is high under conditions of low shear rate and low under conditions of high shear are preferred.

Table 2. Characterization of in-situ gelling systems of sparfloxacin

Parameter Value	F1	F2	F3	F4	F5	F6
% Drug content	98.33	99.5	98.88	100.32	99.73	97.5
pH	6.7	6.8	7	7.4	6.8	7.4
Flow Index 'n'	0.493	0.407	0.361	0.336	0.306	0.116
Consistency Index 'K'dynes/cm^2	3.096	13.749	20.114	49.624	50.582	116.761
Viscosity at 0.1 rpm (cps)	992.2	10 235	11 314	27 548	27 914	39 298

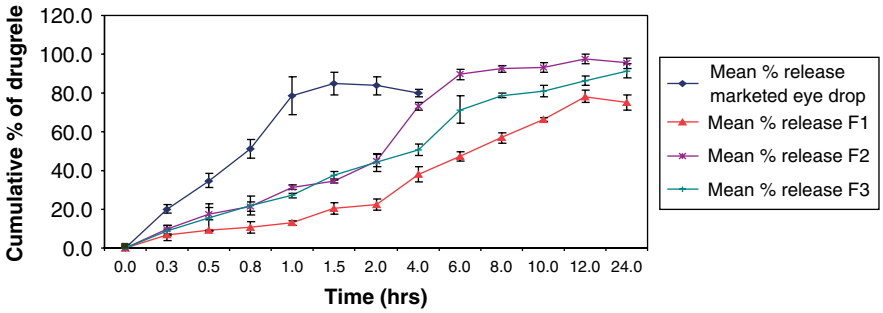


Figure 5. Release profile of sparfloxacin from gel formulations (F1-F3) containing different additives.

The consistency index (k) and the flow index (n) characteristic for each formulation were obtained. The K value of the formulations (F1–F6) was found to be 3.096–116.761. The flow index n is a measure of the deviation of a system from Newtonian behavior ($n = 1$). A value of $n < 1$ indicates pseudoplastic flow or shears thinning; while $n > 1$ indicates dilatants or shear thickening flow. The formulations showed a flow index of 0.116–0.493 indicating pseudoplastic flow behavior as shown in Figure 4.

Table 2 shows that adding 3% CMC polymer (F6) had a viscosity-enhancing effect as revealed by the values of the consistency index K was found to be 116.761 dynes/cm^2. On the other hand, HA polymer also had viscosity-enhancing effect caused by the mucoadhesives property value increased as the concentrations of HA increased where the (K) was increased 43.641, 49.624, and 50.582 dynes/cm^2 for F3, F4 and F5 respectively compared to F1 and F2 where the (K) value was found to be 3.096 and 26.637 dynes/cm^2 *in situ* gels, respectively.

In vitro release study

The cumulative percent of sparfloxacin released from the *in situ* gels formulations (F1–F6) as a function of time is shown in

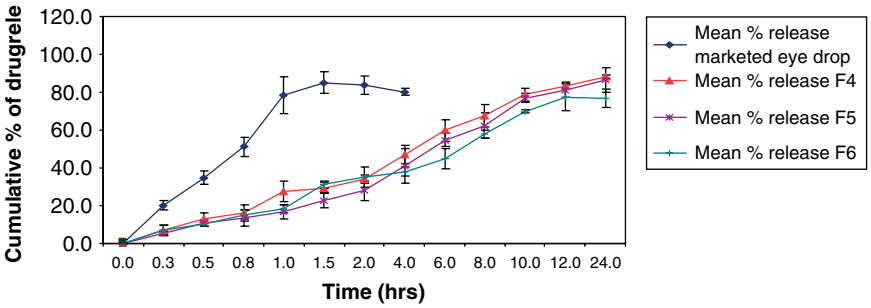


Figure 6. Release profile of sparfloxacin from gel formulations (F4-F6) containing different additives.

Table 3. Release data of sparfloxacin from in- situ gels

Formula	% released	$T_{100\%}$ (hrs)	n	k	Correlation coefficient (R^2)	Release mechanism
marketed eye drops	84.916	1.5	0.537	54.9035	0.788	non Fickian diffusion
F1	78.143	12	0.609	14.78768	0.975	non Fickian diffusion
F2	97.646	12	0.538	27.07073	0.932	non Fickian diffusion
F3	91.3	24	0.542	16.42858	0.945	non Fickian diffusion
F4	88.103	24	0.557	24.82561	0.955	non Fickian diffusion
F5	86.506	24	0.633	20.26749	0.975	non Fickian diffusion
F6	77.193	12	0.546	18.24316	0.949	non Fickian diffusion

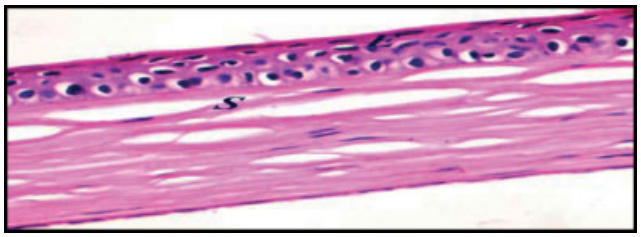


Figure 7. A photomicrograph of a section of corneal tissue of normal rat, (non-infected non-treated) showing normal intact corneal epithelium (E), healthy stroma (S), H&E, X:400.

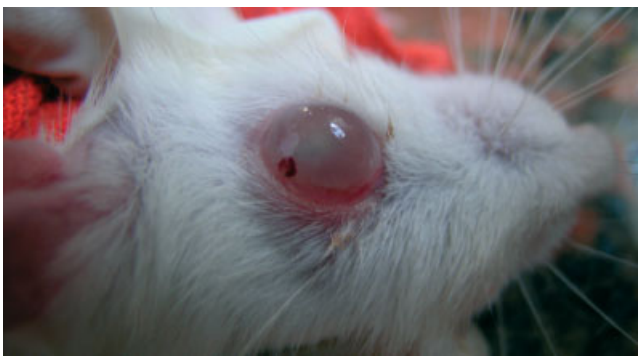


Figure 8. A photomicrograph of infected corneal rat showing induction of keratitis, by *Staphylococcus aureus*.

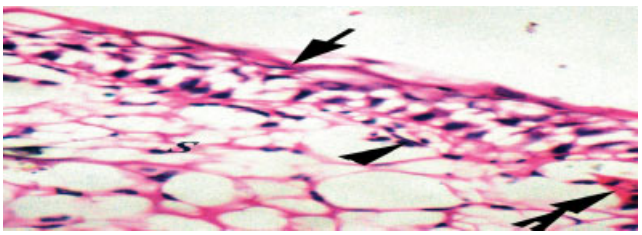


Figure 9. A photomicrograph of a section of corneal tissue of infected non-treated rat as +ve control showing pathology of corneal ulcer, disruption of epithelium layer (arrow), sever edema in stroma (S), with prominent blood vessel (double arrow), and inflammatory cells (Arrowhead) H&E, X: 400.

Figures 5 and 6. F1, F2 and F6 were found to have $t_{100\%}$ to be 12 h if compared to control marketed eye drops that have $t_{100\%}$ to be 1.5 h. Meanwhile the $t_{100\%}$ was 24 h in the case of F3, F4, and F5. Data results also showed that F5 has better sustaining effect amongst all formulations. This may be due to the higher concentration of HA. The results proved that sparfloxacin was sustainedly released, as the content of HA in the copolymer increased due to the mucoadhesive property and high viscosity of HA. The release behavior of each of the six formulae as well as the control was anomalous (non-Fickian) having release exponent (n) values between 0.537 and 0.633 indicating non-Fickian (anomalous) release Table 3.

In vivo histopathological study of the eye cornea

The sections of cornea of group (1) as normal rats stained with H&E demonstrated the histology of normal cornea i.e. thick, intact, non-keratinized stratified squamous epithelium resting on a clear basement membrane. It is formed of five to six cell layers; a single layer of columnar basal cells with basal oval nuclei; intermediate layers of polygonal cells with rounded nuclei; and superficial layers of squamous cells with flattened nuclei. The stroma consisted of regularly arranged collagen bundles and flat keratocytes (corneal fibroblasts) lying in between these bundles, as shown in (Figure 7). The infected cornea of the rat (Figure 8) of non treated group (2) served as positive control, showed by visual inspection redness, and bright green fluorescence. While histological examination showed disruption of epithelium layer, edematous stroma, with prominent blood vessel and lymphatic aggregates with numerous inflammatory cells (leucocytes) invaded the stroma and reduced number of keratocytes in the stroma as shown in (Figure 9). Animals of group 8 (treated with F5), group 7(treated with F4), and group 6 (treated with F3) showed a short period of healing: 3, 4 and 7 days respectively. On the other hand, healing periods for group 5 (treated with F2), group 4 (treated with F1), and group 3 (treated with marketed drops) were 10, 12, and 20 days, respectively, as illustrated in Table 4.

It was obvious from the histopathological examination that group 8 rats treated by formula F5 showed the highest rate of healing. Their corneal tissues appeared almost normal after 3 days of treatment: edema was less, inflammatory reaction could not be seen, and occasional blood vessels were detected. The deep

Table 4. Types of formulations applied and the healing periods

Group	Treated with Formula No.	Period of Healing (Days)
3	Marketed eye drops	20
4	F1	12
5	F2	10
6	F3	7
7	F4	4
8	F5	3

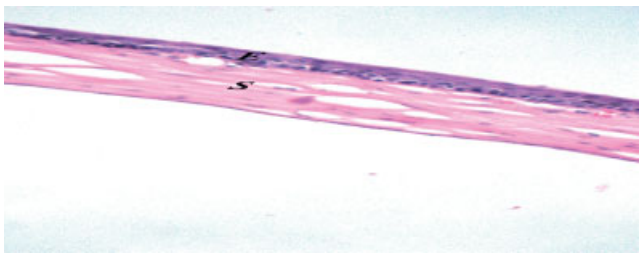


Figure 10. A photomicrograph of a section of corneal tissue exposed to F5- treated group showing healed corneal tissue, where edema is less, no inflammatory cells and occasional blood vessels, H&E, X: 100.

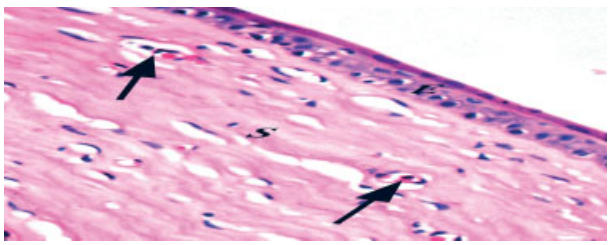


Figure 11. A photomicrograph of a section of corneal tissue after application with F-4, treated rat, showing nearly intact corneal epithelium (E), stroma(S), with occasional blood vessels (arrow), H&E, X: 400.

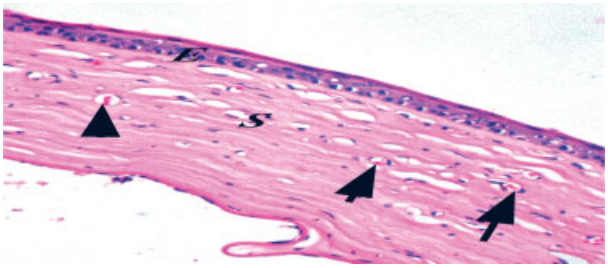


Figure 12. A photomicrograph of a section of corneal tissue after application with F-3, treated rat, showing nearly intact epithelial layer(E), stroma(S) blood vessels is still seen (arrow and arrow head), H&E, X: 200.

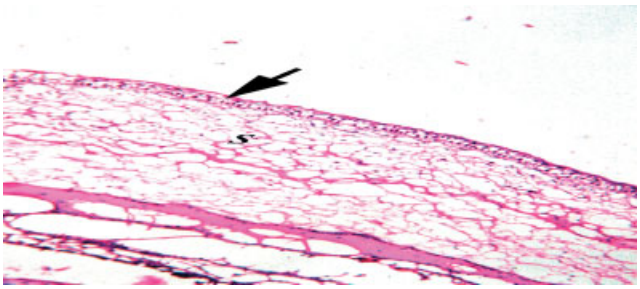


Figure 13. A photomicrograph of a section of corneal tissue after application with F-2, treated rat, showing the ulcer is still present (arrow), with edematous stroma (S), and many congested blood vessels; H&E, X: 100.

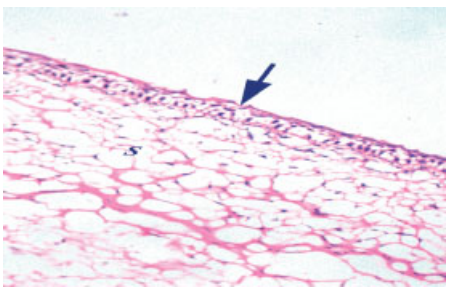


Figure 14. A photomicrograph of a section of corneal tissue after application with F-1 treated rat, showing disrupted epithelium (arrow), moderate edema in stroma (S), with prominent blood vessel, H&E, X: 100.

parts of the stroma appeared compressed. This proves that the application of F5 formula had, to a great extent, ameliorated most of the pathological lesion produced by the infection, where the corneal tissues appeared normal compared to those of the control group as shown in Figure 10. Seven days later, sections in the corneal rats belonging to group 7 (treated with F4), and group 6 (treated with F3), showed healed ulcers (Figures 11 and 12) while those related to group 5 (treated with F2), group 4 (treated with F1), and group 3 (treated with marketed drops) still showed the presence of ulcers (Figures 13–15).

From the aforementioned results, it can be concluded that the different formulae used to overcome the pathological alterations produced by bacteria infections varied depending on the effectiveness of the formulation as well as healing process. They were arranged as F5, F4, and F3 in descending order. F5 was excellent, containing 0.5% HA which helps tissue regeneration.

Figure 15. A photomicrograph of a section of corneal tissue after application with marketed eye drops, treated rat, showing disrupted epithelium (arrow), moderate edema in stroma (S), with prominent blood vessel, H&E, X: 400.

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