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Preparation and characterization of ophthalmic in situ forming gels containing forskolin

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ABSTRACT

Open angle glaucoma (POAG) is characterized by a persistent high intraocular pressure. Many conventional drug formulations are in use for its treatment, but they are associated with relevant side effects and a large loss of bioavailability because of dilution by tears and rapid drainage. Forskolin (FOR) is an herbal product from Indian traditional medicine that showed to be potentially useful in POAG treatment. Polymeric solutions forming in situ gel structures are suitable candidates to prolong the contact time of a drug with the corneal surface, thus promoting its adsorption in the eye anterior portion. In this work FOR was loaded in liquid formulations containing different polymeric materials and concentrations, able to undergo a liquid-to-gel phase transition caused by local stimuli on the eye surface, like variations of temperature, pH or presence of ions in the tear fluid. Gelling capacity studies showed that thermo-sensitive and ion-sensitive systems were the most compatible formulations for FOR. Rheological studies confirmed the liquid-gel transition phase, and stability studies showed that these systems are stable up to six months. Selected formulations were submitted to an ex-vivo pharmacokinetic study and an in vivo biodistribution test, that indicated as this administration approach can prolong the time of contact of the drug with the anterior eye portion.

Keywords: *Ocular dosage forms; eye-drops; controlled drug delivery; biopolymers; glaucoma.*

1. INTRODUCTION

Forskolin (FOR, Fig. 1) is the most abundant labdane diterpenoid present in the root extract of Coleus foskohlii (Willd.) Briq., an aromatic herb grown especially in India and South-East Asia, member of the Lamiaceae family. FOR is a reversible activator of adenylyl cyclase (AC) [1], which explains the wide range of pharmacological effects and the use of this plant since ancient time in traditional Indian medicine for many applications, such as treatment of hypertension, asthma, eczema, respiratory disorders, and glaucoma [2, 3].

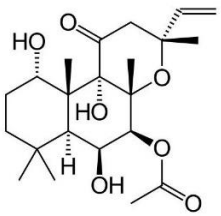

Figure 1. Structure of forskolin.

Primary open angle glaucoma (POAG) is a particular condition of glaucoma characterized by a persistent high intraocular pressure. Current therapies mainly use beta-blockers and prostaglandin analogs: the former drugs possess severe systemic side effects at the cardiac and pulmonary level, while the second class of compounds causes an undesired increased iris pigmentation. The need thus emerges for alternative active agents, whose efficacy is associated with a reduced local and systemic toxicity. Among those, FOR has shown to reduce IOP in clinical trials on POAG patients [4-6] and to restore retinal damage induced by ocular hypertension [7]. FOR can cross cell membrane and activates AC without interacting with cell surface receptors, but through a direct action of the diterpene moiety on the catalytic subunit of the enzyme. FOR requires the presence of guanine nucleotide-binding protein (G8 protein) for maximal stimulation of the enzyme. Activation of AC in turn increases the intracellular level of cyclic adenosine monophosphate (cAMP) that, among the other effects, at the level of ciliary epithelium causes a reduction of aqueous humor inflow and a lowering of intraocular pressure (IOP) [8, 9].

Conventional ophthalmic dosage forms, like eye drops, suspensions or ointments, share various problems in terms of low bioavailability, short duration of the activity and, in the last case, also blurred vision. These limitations are amplified by the induction of tear production and turnover, together with unproductive eye drainage and adsorption, so that only a limited fraction of the applied drug can reach the target area or tissues.

To enhance the efficacy of drugs, by prolonging the contact time with corneal surface, various ocular drug delivery systems have been investigated, among which colloidal nanocarriers (liposomes, nanoparticles or nanosuspensions) and ocular polymeric inserts [10-12]. Many of these strategies are experiencing a huge attention also by pharmaceutical companies [13-16]. These systems allow to improve drug local bioavailability and prolong their activity, thus allowing to reduce the dose frequency and improve therapeutic compliance.

In recent years, in situ forming gel systems have been proposed as a strategy to enhance the corneal retention time and permeation of various drugs, including agents used in the treatment of glaucoma [17-20]. These products are formed by polymer solutions in an aqueous medium that undertakes a reversible liquid-gel phase transition under specific physical or chemical stimuli. These formulations can be instilled as conventional drops and quickly gelify when coming in contact

with the eye surface. Gelation can be induced by various stimuli, such as temperature, pH value and electrolyte compositions of the tear fluid. Different kinds of polymers can be therefore used to realize these in situ forming gels, in accordance with their physico-chemical nature and sensitivity [21, 22], and in several cases associations of polymers sensitive to different stimuli, e.g. temperature and pH or ionic strength have been proposed to improve the gelling capacity of the formulation after the application.

In this first study, we produced and characterized three different stimuli-responding gelling systems loaded with FOR. A first series of formulations were based on poloxamers (common trade names: Synperonics®, Pluronics®, Kolliphor®), nonionic triblock copolymers composed of a central hydrophobic chain of poly(oxypropylene) flanked by two hydrophilic chains of poly(oxyethylene). These thermo-responsive materials exhibit reverse thermal gelation at determined temperatures and within specific concentration ranges [23-27]. A second series of batches used carbomers (Carbopol®), a family of acrylic acid polymers usually cross-linked with poly(alkenyl)ethers or divinyl glycol; they contain a carboxyvinyl group and therefore show a liquid-gel transition in aqueous solutions in response to pH changes [28-31]. A third series of gelling systems was made using gellan gum (e.g., Gelrite®), an exocellular polysaccharide of microbiological origin, produced by Sphingomonas elodea. It consists of tetrasaccharide repeating units (glucose, glucuronic acid, and rhamnose, in a 2:1:1 ratio), that can form a viscoelastic gel structure in the presence of cations, and especially bivalent ones [32,33].

Selected formulations among those prepared were subjected to ex-vivo and in vivo tests to evaluate the distribution of FOR in the eye tissues. The technological and biological outcomes of this preliminary study will allow us to select one or more among the most performing gels for an in vivo evaluation of the pharmacokinetics and pharmacological profile of FOR after ocular application and for the possible association of two or three kinds of in situ gelling polymers in a single, optimized and highly performing formulation.

2. EXPERIMENTAL SECTION

Materials. FOR (purity: 98%) was purchased from Molekula srl (Rimini, Italy); gellan gum was purchased from Thermo Fisher (Kandel) GmbH (Karlsruhe, Germany); hydroxypropyl-methylcellulose (HPMC, Mantrocel®) was purchased from Gustav Parmentier GmbH (Frankfurt, Germany); Cremophor® A25 and disodium EDTA were purchased from Sigma-Aldrich srl (Milan, Italy); Pluronic® F127 and F68 were kindly gifted by BASF (Cesano Maderno, Italy); Carbopol® 974P NF was gifted by Lubrizol Advanced Materials Europe BVBA (Bruxelles, Belgium). All formulations were made using water for injection containing 0.002% (w/v) NIG, kindly gifted by Sooft Italia spa (Fermo, Italy). Simulated tear fluid (STF) was prepared containing 200 mg of sodium bicarbonate, 670 mg of sodium chloride and 8.3 mg of dehydrate calcium chloride in 100 ml of water for injection (corresponding to 1.11 mEq/L calcium, 142 mEq/L sodium, 116.7 mEq/l chloride, 27.2 mEq/L bicarbonate). The pH was adjusted to 7.4 using 0.1 N HCl or NaOH.

To obtain the thermo-sensitive gels, mixtures of Pluronic® F-127 (PF127) and F-68 (PF68) (Table 1) were dispersed in preserved water under mechanical stirring for 24 h, at room temperature (r.t.), to allow the complete dissolution of the polymers. The final solution was then stored at 4°C. The selection of the different percentages of PF127 and PF68 was based on literature data [25] and after preliminary tests made in our lab.

After an evaluation of the gelling properties of these blank systems at 35°C (see below), two Pluronic® mixtures were selected to incorporate the drug. FOR was added to the initial polymer dispersion at either 0.5 or 1% (w/v) final concentration. Table 4 gathers the composition and properties of the drug-loaded formulations.

To produce the pH-sensitive gels, mixtures of different percentages of Carbopol® 974P NF, HPMC and EDTA, if required, were dissolved in preserved water under mechanical stirring for 4 h at r.t. The mixture was then stored at r.t. in closed glass vials. Table 2 reports the composition of the blank formulations produced and tested for the gelation capacity at pH 7.4, to select the systems to be added with FOR. The drug was added to the initial dispersion of the polymers (Table 5).

Ion-sensitive *in situ* forming gels were produced by dissolving different percentages of gellan gum in preserved water under constantly mechanical stirring for 1 h at 85°C [12], in order to allow its complete dissolution. the mixtures were stored at r.t. Table 3 summarizes the composition of the blank formulations obtained. The most interesting batches were G3B00 and G3D00; therefore, in these systems, the addition of Cremophor® A25 was evaluated, as a surfactant agent able to increase FOR solubility and also based on its high ocular tolerability [28]. The inclusion of FOR in selected formulations was made by adding the drug to the other powders before heating. The final formulations (Table 6) were stored at r.t.

Physicochemical characterization. The prepared formulations were tested for clearness, pH value, rheological behavior, gelling capacity, stability, and *ex-vivo* and *in vivo* ocular biodistribution tests.

pH measurement. The pH of each formulation was measured using a VWR-9100 pH-meter, that was calibrated using standard solutions at pH 4.0, 7.0, and 10.0 ± 0.01 at 25°C. All formulations were tested in triplicate and the mean value was calculated.

Gelling studies. One ml of each formulation was placed in a clean glass test tube. The gelling capability for temperature sensitive *in situ* forming gels was evaluated under mild mechanical stirring, while temperature was gradually raised (2°C/min) by an electronic thermo-controller (ETS-D3, IKA-Werke GmbH & Co. KG, Staufen, Germany), registering the gelation temperature. The test was repeated for each formulation after contact with STF (200 µl added to 1 ml of formulation), to measure the sol-gel temperature transition under physiological conditions. The ability for pH- and

ion-sensitive polymer solutions to form gels was evaluated similarly upon contact with STF, under mild stirring at r.t.

Dynamic viscosity measurement. The rheological properties of blank ion-dependent systems were evaluated, before and after addition of STF, using a Bohlin instrument (CVO) programmable rheometer . Firstly, the ion-dependent formulation (2 ml) was placed on the cone-plate holder (4° angle, 4 mm diameter) and the angular velocity (shear rate) was gradually increased. The viscosity was evaluated on the same batch using 2 ml of formulation mixed with STF (400 µl). The two measurements were compared, in order to evaluate the increase in dynamic viscosity induced by the presence of STF (Figure 2).

The influence of increasing percentages of surfactant (Cremophor® A 25) on the rheological behavior of gellan gum was also observed. Three ion-sensitive formulations containing 0.2, 0.3 and 0.4% Cremophor® A25 were tested (Table 3). Figure 3 reports the results of rheological results; statistical significance (Student t-test) was set at $p<0.05$.

Storage test. Each formulation was stored under suitable conditions (4°C for thermo-sensitive systems, at r.t. for the other two systems) and at specific times (1, 3 and 6 months) appearance, pH value and gelling ability was measured and compared with the initial values.

***In vitro* dissolution test.** The dissolution of FOR from an aqueous suspension or from three representative gelling systems was investigated by a dialysis method. Bags made with Spectra/Por® 3 cellulose acetate membranes (MWCO: 3.5 kDa), previously soaked for 6-8 h in the dissolution medium (STF), were filled with 1 ml of each formulation and immersed in a beaker containing 25 ml of freshly prepared STF (pH 7.4) added with 1% (w/v) PEG 40 to improve FOR solubility and provide sink conditions. The system was maintained at 35 ± 1 ˚C under magnetic stirring at 50 rpm. At determined time intervals, 2-ml samples were withdrawn and replaced with a corresponding volume of the dissolution medium. FOR dissolution test was realized under analogous conditions, placing in the dialysis bag 1 ml of a drug suspension (0.5%, w/v). Each sample was analyzed by UV spectrophotometry at 226 nm (Genesys 10 UV scanning spectrophotometer, ThermoFisher Scientific, Italy); drug concentration values were calculated by means of a calibration curve of pure FOR in water/ethanol (1:1 by volume, linear in the range 0.05–1 mg/ml; $r^2=0.9992$) and corrected for dilution effect.

***Ex-vivo* pharmacokinetic assay.** Selected formulations were tested in a model consisting of an explanted pig eye, kept horizontally on a plastic holder. The test formulations (200 µl) were applied using a plastic ring placed upon the cornea, to contain the product. Each test was carried out in triplicate. After 15 min, the aqueous humor and the whole cornea from each treated eye were collected and treated as described below for the analytical determination of FOR.

***In vivo* biodistribution assay.** Three male New Zealand albino rabbits were used for each formulation to test. A drop (50 µl) of an 1% (w/v) FOR suspension in PBS (pH 7.4) or gelling formulations **G1B1a** and **G2G1** was instilled in the lower cul-de-sac of each eye at time 0, 2, 4 and 6 h. At the 7th hour from the beginning of the assay, the aqueous humor was taken and stored at -20°C until the analytical determination of drug concentration was made.

To determine the concentration of FOR in biological samples, aqueous humor samples were extracted with ethyl acetate. Corneal samples were added to 500 ml of ammonium acetate buffer (20 mM, pH 7.2), homogenized, and extracted with ethyl acetate. After vortex mixing for 10 min and centrifugation at 4,000 rpm at +4°C for 15 min, aliquots of 2.5 ml of the organic phase were transferred into a single glass tube and dried under vacuum at 50°C using a Büchi vacuum system. The residues were reconstituted with water/acetonitrile. The tubes were then vortex-mixed and centrifuged. Liquid chromatography mass spectrometry (HPLC-MS/MS) analysis was performed using an Agilent instrumentation. The final organic extracts were transferred into an autosampler vial, and 10 µl were injected into a Phenomenex Kinetex C-18 column (4.6 mm i.d. x 50 mm length, 2.6 µm beads) was used to perform the chromatographic isocratic analysis (25% 5 mM ammonium acetate – 0.1% formic acid/75% methanol) at a flow rate of 0.1 ml/min. Retention times of FOR were about 2.45 min; total run time was 3 min. MS detection used an Agilent Technologies 6410 Triple Quadrupole LC/MS instrument with atmospheric pressure ionization/electrospray ionization (API/ESI) source and multiple reaction monitoring (MRM) (375.3 to 297.2 m/z mass transition) operated in the positive ion mode.

Calibration curves of FOR in the cornea and aqueous humor were made by plotting the ratio of the area of the compound (y) against the analyte concentration (x). The analysis gave linear plots in the range 500-2500 ng/ml for corneal specimens and 10-150 ng/ml for aqueous humor samples.

Statistical analysis. Experimental results are expressed as the mean ± S.D. One-way ANOVA analysis was applied to the results; statistical significance (Student *t*-test) was set at $p<0.05$.

3. RESULTS SECTION

Gelling capacity and physical parameters. Three groups of blank (unloaded) in situ gelling systems, respectively based on thermo-sensitive (batches **G1**), pH-sensitive (batches **G2**) and ion-sensitive polymers (batches **G3**) (Tables 1-3) were preliminarily evaluated to ascertain the best composition in terms of gelling capacity. Gelation was evaluated both upon contact of 1 ml of each formulation with water or simulated tear fluid (STF) (200 µl). For batches G1 the assay was carried out at 35°C, i.e. the physiological temperature of the eye surface, while for other two series the pH value and ion concentration proper of STF were benefitted to induce the gelation of the polymers. All formulations were prepared using water containing N-hydroxymethylglicinate (NIG) (0.002% by weight) as a non-toxic preserving agent [34].

Among the pH-sensitive formulations (Table 2), disodium EDTA was added in two batches to evaluate its influence on the behavior of the polymer solutions. For this sequestering action, in fact, EDTA is able to prevent the Carbopol®-ion interaction and promote the gelation. Furthermore, EDTA is known and used in

commercial ophthalmic products as a promoter of drug corneal penetration of poorly soluble drugs [35]. In the same systems, HPMC was added at various percentages to modulate the excessive viscosity of Carbopol® solutions.

Table 1. Composition (%, w/v) and gelling behavior of blank thermo-sensitive *in situ* gelling systems based on mixtures of Pluronic® F-127 (PF127) and Pluronic® F-68 (PF68).

Batch	PF127	PF68	Gelling capacity at 35°C[a]
G1A0	20	5	—
G1B0	25	5	++
G1C0	25	3	—
G1D0	20	10	—
G1E0	30	5	—
G1F0	15	5	—
G1G0	25	2.5	++
G1H0	10	5	—
G1I0	20	2.5	—
G1L0	16.5	0	—

a: — No gelation; ++ immediate formation of a gel which persisted fora long time.

Table 2. Composition (%, w/v) and gelling behavior of blank pH-sensitive *in situ* gelling systems.

Batch	Carbopol	HPMC	EDTA	Gelling capacity at pH 7.4[a]
G2A0	0.1	0.4	-	—
G2B0	0.3	1	-	—
G2C0	0.5	1	-	—
G2D0	0.2	0.1	-	—
G2E0	0.3	0.1	-	+
G2F0	0.4	0.1	-	—
G2G0	0.3	0.1	0.02	—
G2H0	0.5	0.1	-	—
G2I0	0.3	0.2	0.02	+
G2L0	0.3	0.2	-	+

a: — No gelation; + a gel was formed, that lost consistency after few minutes.

In a second phase, selected formulations were loaded with FOR at two different drug concentrations (0.5% or 1%, w/v) (Tables 4-6). In particular, among the Pluronic®-based systems two mixtures (batches **G1B** and **G1G**, Table 1) showed to undergo a sol-gel transition when warmed at 34°C, whereas for the other compositions only partial or no gelation was registered. Among the pH-dependent gelling mixtures, batches **G2E** and **G2L** (Table 2) formed a gel upon contact with at pH 7.4 buffer. At lower or higher percentages of Carbopol 974P NF (Table 2) gelation was less evident also after 30 min of contact with the buffer solution. Finally, among the gellan gum formulations batches **G3B** and **G3C** were chosen because they showed the best ability to form a permanent gel in the presence of STF (Table 3).

Table 3. Composition (%, w/v) and gelling behavior of blank ion-sensitive *in situ* gelling systems.

Batch	Gellan gum	Cremophor® A 25	Gelling capacity upon contact with STF[a]
G3A00	0.2	0	—
G3B00	0.3	0	—
G3B01	0.3	0.2	++
G3B02	0.3	0.3	—
G3B03	0.3	0.4	++
G3C00	0.4	0	—
G3C01	0.4	0.2	++
G3D00	0.5	0	++
G3D01	0.5	0.2	+++
G3D02	0.5	0.4	+++
G3F00	0.6	0	+++

a: — No gelation; ++ immediate formation of a gel which persisted for a long time; +++ formation of a highly viscous gel.

The best performance among these solutions was observed at a gellan gum concentration of 0.3 and 0.4% (w/v), while at higher percentages of polymer an excessively viscous gel was produced, unsuitable for ocular application as eye-drops.

The produced systems were characterized for visual appearance (transparency), pH, gelling capacity and duration, viscosity and mid-term physico-chemical stability.

Table 4. Composition (% w/v), pH and appearance of temperature-sensitive formulations containing FOR. The gelling temperature (T sol-gel) was determined upon contact of each formulation (1 ml) with distilled water or STF (200 µl).

Batch	Composition			Gelling capacity (T sol-gel,°C)		pH	Appearance
	PF127	PF68	FOR	water	STF		
G1B1a	25	5	1	25.7	34.0	7.20	opalescent
G1B1b	25	5	0.5	27.7	37.0	6.78	opalescent
G1G1b	25	2.5	0.5	22.9	33.3	6.82	opalescent

Table 5. Composition (% w/v), pH and appearance of pH-sensitive formulations loaded with FOR (1%, w/v). The gelling capacity was evaluated upon contact of the formulation (1 ml) with STF (200 µl) at room temperature.

Batch	Carbopol	HPMC	EDTA	FOR	pH	Gelling capacity with STF [a]	Appearance
G2E1	0.3	0.1	-	1	3.3	++	milky
G2G1	0.3	0.1	0.02	1	3.4	++	milky
G2I1	0.3	0.2	0.02	1	3.7	++	milky

a: ++ immediate gelation, that persisted for some minutes.

Table 6. Composition (% w/v), pH value and appearance of FOR-loaded ion-sensitive formulations. Gelation was evaluated upon contact of the formulation (1 ml) with STF (200 µl) at room temperature.

Batch	Gellan gum	Cremophor A 25	FOR	pH	Gelling capacity with STF [a]	Appearance
G3B1.2	0.3	0.3	0.5	6.87	—	milky
G3C1.1	0.4	0.2	0.5	6.65	++	milky
G3F1.2	0.6	0.3	0.5	6.62	++	milky

a: — No gelation; ++ immediate gelation, that persisted for some minutes.

Table 7. Gelling temperature (°C) and pH values of fresh and aged thermo-sensitive formulations containing FOR, upon contact of 1 ml of each formulation with 200 µl of STF.

	Time 0			3 months			6 months		
Batch	Water	STF	pH	Water	STF	pH	Water	STF	pH
G1B1a	25.7	34.0	7.20	25.5	35.2	6.90	27.0	>40	6.90
G1B1b	27.7	37.0	6.78	28.5	37.0	6.78	29.1	35.0	6.80
G1G1b	22.9	33.3	6.82	22.0	33.3	6.82	22.5	27.5	6.38

Stability studies. The produced formulations were stored at 4°C for thermo-sensitive formulations and at 25°C for the pH-sensitive and ion-sensitive systems. After 3 and 6 months, each formulation was re-evaluated for gelling capacity, pH value and physical appearance. Tables 7-9 gather these experimental data.

Table 8. Gelling temperature (°C) and pH values of fresh and aged pH-sensitive formulations containing FOR. One ml of each formulation was poured in contact with 200 µl STF at room temperature.

	Time 0		3 months		6 months	
Batch	Gelling capacity[a]	pH	Gelling capacity	pH	Gelling capacity	pH
G2E1	++	3.31	++	3.52	++	3.90
G2G1	++	3.41	++	3.42	+	3.81
G2I1	++	3.70	++	3.41	+	3.35

a: + slow gelation; ++ immediate gelation, persisting for some minutes.

Table 9. Gelling temperature (°C) and pH values of fresh and aged ion-sensitive formulations containing FOR. One ml of each formulation was poured in contact with 200 µl STF at r.t.

	Time 0		3 months		6 months	
Batch	Gelling capacity[a]	pH	Gelling capacity	pH	Gelling capacity	pH
G3B1.2	—	6.87	—	6.90	—	7.40
G3C1.1	++	6.65	+	6.72	+	7.10
G3F1.2	++	6.62	+	6.61	—	7.00

a: — no gelation; + slow gelation; ++ immediate gelation, persisting for some minutes.

Rheological studies. The viscosity of one gellan gum solution (batch **G3B01**), both void and containing FOR was assessed. Measurements were made both in the presence of water or STF, in order to evaluate the changes in viscosity induced by the presence of simulated tears. The analysis stressed the gelling capacity observed *in vitro*. The viscosity of sample solutions was measured at different angular velocities before and after the addition of STF. The formulations were similar to Newtonian fluids in non-physiological condition, whereas they showed pseudoplastic properties in physiological conditions, i.e. upon contact with STF (37°C and pH 7.4) (Fig. 2).

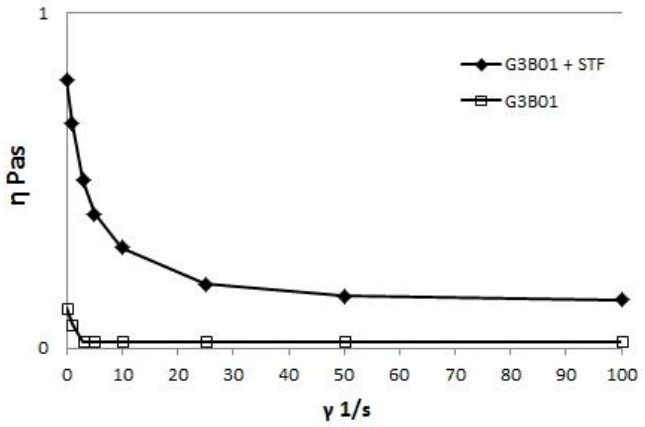

Figure 2. Dynamic viscosity at 25°C of batch G3B01 upon contact with distilled water or STF.

Moreover, the influence of the surfactant (Cremophor® A25) in these formulations was assessed. By assaying a fixed gellan gum concentration (0.3%, w/v) and three different surfactant concentrations (0.2, 0.3 and 0.4%, w/v), rheological analyses indicated that the viscosity slightly decreased with increasing the surfactant concentration. However, the surfactant reduced the turbidity of the preparation, helping to dissolve the poor water soluble FOR. The effect of Cremophor® A 25 concentration is shown in Figure 3.

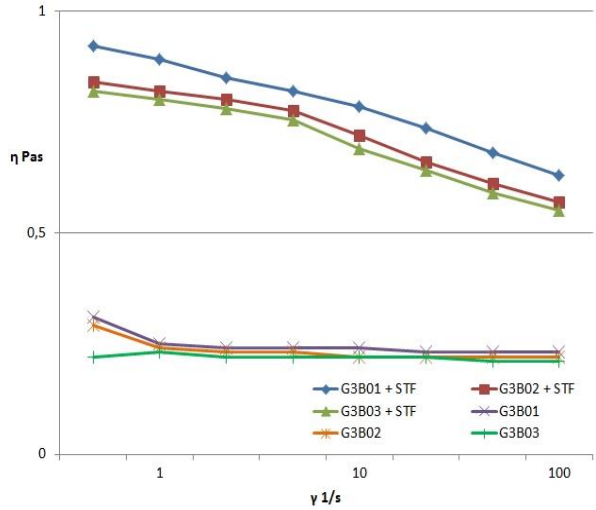

Figure 3. Effect of Cremophor® A 25 concentration upon the viscosity of gelled ion-dependent systems.

***In vitro* release studies.** Figure 4 shows the dissolution profile of FOR from an aqueous suspension and the drug release curved from three different gelling systems.

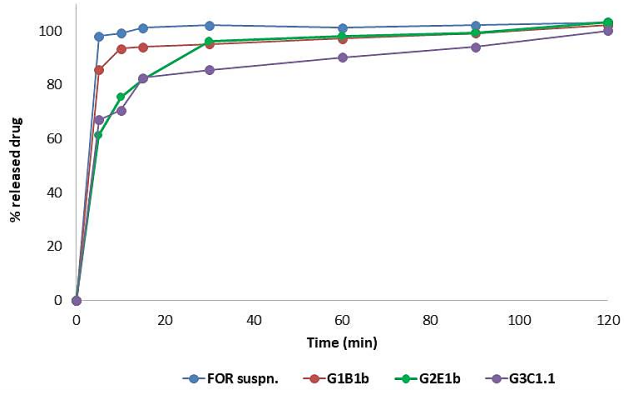

Figure 4. Dissolution profile at 35°C of FOR from a 0.5% (w/v) aqueous suspension or from three representative gelling formulations. The receiving phase was STF, pH 7.4.

While the neat drug quickly dissolved in the receiving medium (STF, pH 7.4) within the first minutes of the assay, the experimental findings indicated that the different gelling systems release gradually the dispersed drug. In particular, the ion-dependent (batch **G3C1.1**) formulation released the entire amount of FOR within 60 min, while for the pH-dependent (**G2E1**) and

the temperature-dependent (batch **G1B1b**) formulations the complete release of the drug was achieved in about 30 min.

Biological experiments. The results of *ex-vivo* studies are illustrated in Figure 5. After a 15-min contact of an 1% (w/v) suspension of FOR in PBS with the explanted pig eye, the drug was detected both in the corneal tissues and, at a 7-fold higher concentration, in the aqueous humor, suggesting a quick diffusion through the cornea.

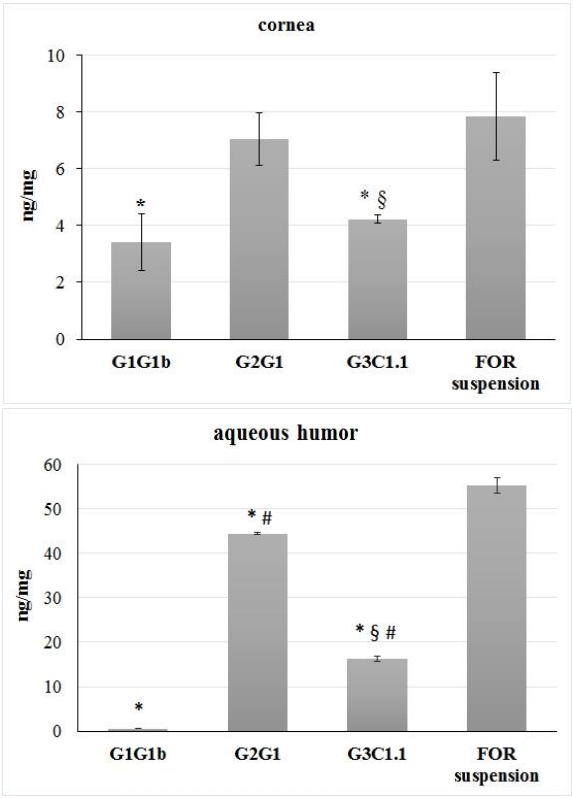

Figure 5. Measured levels (ng/mg of tissue sample) of FOR in the cornea (up) and aqueous humor (down) after 15 min contact of a drug suspension in PBS or three of the prepared *in situ* gelling formulations with explanted pig eye (see Tables 4-6 for composition). Student *t*-test: *p<0.05 vs FOR aqueous suspension; § p<0.05 vs G2G1; # p<0.05 vs G1G1b.

Among the tested gelling systems, in particular the pH–sensitive system (**G2G1**) ensured a diffusion of the drug in the cornea and aqueous humor comparable to the aqueous suspension of the drug. After topical application of the other two systems, lower drug concentrations were found in ocular tissues. Using the thermo-sensitive formulation **G1G1b**, no drug was detected beyond the cornea, maybe as a consequence of a scarce diffusion of FOR out of this polymeric solution on the eye surface.

In the *in vivo* studies, FOR concentration was evaluated in the aqueous humor of rabbits after installation of formulations **G1B1a** and **G2G1**, both containing 1% FOR (w/v), representative of thermo-sensitive and pH-dependent systems, respectively. For comparison, a 1% (w/v) suspension of FOR in PBS was used. As shown in Figure 6, the pH-dependent **G2G1** formulation showed in vivo a similar behavior than the aqueous suspension of the neat drug, while with the Poloxamer® based gelling system a lower concentration of FOR was reached.

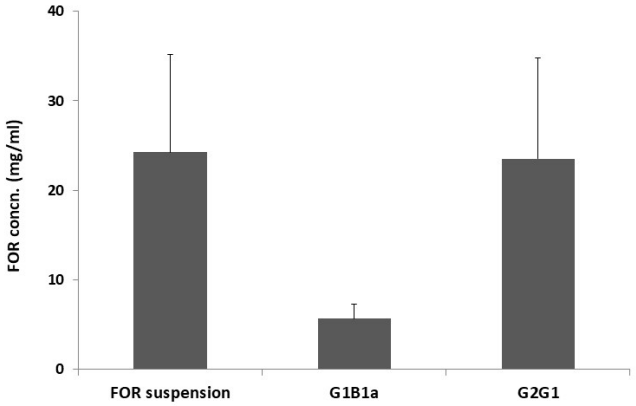

Figure 6. Concentration of FOR in rabbit aqueous humor after topical application of a 1% suspension of the drug in PBS or two of the prepared gelling formulations (G1B1a and G2G1) (see Tables 4 and 5 for composition).

4. CONCLUSIONS

The results of these studies suggest that *in situ gelling systems* are promising for controlled drug release of FOR on the corneal surface. Three different systems of *in situ* forming gels were developed. The gelling capacity studies have shown that thermo-sensitive and ion-sensitive formulations were the most compatible systems for this drug. Rheological studies confirmed the liquid-gel transition phase, and stability studies showed that the systems are stable up to six months.

Both *ex-vivo* and *in vivo* assays showed that these systems can prolong the time of contact with the anterior eye portion. Although the above *ex-vivo* and *in vivo* assays were largely different in terms of animal model used (e.g., the pig cornea is known to have an almost double thickness than the human one), volume of applied formulation, duration of the experiment and, not less important, the horizontal set-up of the explanted eye model, it is interesting to observe that both models indicated that the pH-sensitive formulations (batches **G2**, Table 5) were able to produce a drug concentration in the cornea and/or anterior eye structures comparable with a neat drug suspension, whereas the Poloxamer®-based thermo-sensitive systems **G1** were in both cases unable to ensure a rapid release of the dispersed drug and thus its diffusion in the cornea and aqueous humor.

Deeper *in vivo* experiments are required to validate these preliminary findings, but the reported results are however encouraging for a further pharmacokinetic and pharmacological evaluation of selected FOR-loaded technological systems. In particular, the effect of gelling polymers on the length of the permanence of FOR on the corneal surface, and thus on its prolonged diffusion in the eye anterior chamber would be assessed.

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