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Optimization and evaluation of thermoresponsive diclofenac sodium ophthalmic in situ gels

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ABSTRACT

This work was conducted to optimize and evaluate Pluronic F127-based thermoresponsive diclofenac sodium ophthalmic in situ gels (DS in situ gel). They were prepared by cold method and investigated their physicochemical properties i.e., pH, flow ability, sol–gel transition temperature, gelling capacity and rheological properties. An optimized formulation was selected and investigated its physicochemical properties before and after autoclaving, eye irritation potency in SIRC cells and rabbits. In vivo ophthalmic absorption was performed in rabbits. It was found that physicochemical properties of DS in situ gels were affected by formulation compositions. Increment of Pluronic F127 content decreased sol–gel transition temperature of the products while increase in Pluronic F68 concentration tended to increase sol–gel transition temperature. In this study, Carbopol 940 did not affect sol–gel transition temperature but it affected transparency, pH, and gelling capacity of the products. The optimized formulation exhibited sol–gel transition at 32.6 ± 1.1 °C with pseudoplastic flow behavior. It was lost diclofenac sodium content during autoclaving. However, it was accepted as safe for ophthalmic use and could increase diclofenac sodium bioavailability in aqueous humor significantly. In conclusion, the optimized DS in situ gel had potential for using as an alternative to the conventional diclofenac sodium eye drop. However, autoclaving was not a suitable sterilization method for this product.

1. Introduction

Eye is one of the challenging organs for drug delivery because its unique anatomy restricts drug absorption into the deep tissues. Although various ophthalmic drug delivery systems such as inserts, ointments and suspensions have been developed to overcome this problem, they have not been accepted by patients due to several drawbacks i.e., difficult administration of inserts, blurred vision from use of ointments and dosage heterogeneity of suspensions (Nanjawade et al., 2007). Consequently, more than 90% of marketed ophthalmic formulations are still in a form of an eye drop of water soluble drugs (Araújo et al., 2009). The main reason for using the eye drop is simple instillation into the eye with accuracy doses (Ludwig, 2005; Qi et al., 2007). However, this conventional system could not be considered as an optimum formulation for treatment of eye disorders due to the rapid precorneal elimination by protective mechanisms of the eye such as blinking reflex, lacrimal fluid dilution and nasolacrimal duct drainage (Nanjawade et al., 2007). Nowadays, a major progress in development of ophthalmic formulations has been performed by the ophthalmic gel technology in the development of droppable gels called an "in situ gel" which consists of certain polymers undergoing sol–gel phase transition by an induction of environment conditions, for example: pH (Srividya et al., 2001; Wu et al., 2007), specific ions (Ludwig, 2005) and temperature (Wei et al., 2002; Dumortier et al., 2006; Escobar-Chávez et al., 2006).

In particular, a thermoresponsive in situ gel, an ophthalmic product vehicle responding to a shift in temperature, possesses liquid characteristic at low temperature and becomes gel when comes

Abbreviations: AUC, area under curve; °C, degree Celsius; C_{max}, maximum concentration; cm, centimeter; CO_2, carbon dioxide; DS in situ gel, thermoresponsive diclofenac sodium ophthalmic* in situ gel; F127, Pluronic F127; F68, Pluronic F68; g, gram; G', storage modulus; G'', viscous modulus; HCl, hydrochloric acid; H_2O, water; HPLC, high-performance liquid chromatography; i.e., id est; K_a, absorption rate constant; K_e, elimination rate constant; kg, kilogram; %LA, percentage labeled amount; M, molar; MEC, minimum effective concentration; min, minutes; mg, milligram; ml, milliliter; mm, millimeter; MTT, methylthiazolydiphenyl-tetrazolium bromide; N, normality; n.d., not determined; ng, nanogram; nm, nanometer; Pa, Pascal; PEO, poly(ethylene oxide); PPO, poly(propylene oxide); RH, relative humidity; s, second; SD, standard deviation; STE, short time exposure; STF, simulated tear fluid; $t_{1/2}$, half life; T_{max}, time to reach C_{max}; $T_{sol-gel}$, sol–gel transition temperature; UV, ultraviolet; w/w, weight by weight; %, percentage; μl, micro liter.

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Table 1
Formulation compositions of thermoresponsive diclofenac sodium ophthalmic in situ gels.

Ingredients	Content of ingredients in each formulation (%, w/w)							
	F127-14	F127-20	F127-26	F127-20 + F68-8	F127-20 + F68-11	F127-20 + F68-14	F127-20 + F68-11+C0.1	F127-20 + F68-11+C0.3
Diclofenac sodium	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Pluronic F127 (F127)	14	20	26	20	20	20	20	20
Pluronic F68 (F68)	0	0	0	8	11	14	11	11
Carbopol 940	0	0	0	0	0	0	0.1	0.3
Benzalkonium chloride	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Phosphate buffer qs ad	100	100	100	100	100	100	100	100

into contact with a certain temperature defined as a sol–gel transition temperature, which could be determined by using a number of techniques such as spectroscopy, differential scanning calorimetry and rheology (Klouda and Mikos, 2008). It has been suggested that a good ophthalmic thermoresponsive in situ gel should have sol–gel transition temperature higher than room temperature and form gel at precorneal temperature (35 °C) to avoid keeping in a fridge before administration which may lead to eye irritation from use of cold eye drops (Wei et al., 2002; Ma et al., 2008).

One of well-known polymer types possessing thermoresponsive behavior is Pluronics, so called Poloxamers. They are a triblock copolymer poly(ethylene oxide)-b-poly(propylene oxide)-b-poly(ethylene oxide) (PEO–PPO–PEO) showing amphiphilic behavior due to hydrophilic ethylene oxide domains and hydrophobic propylene oxide domains. The gelation mechanism of Pluronics could be explained by the changes in micellar structure as a function of concentration and temperature (Klouda and Mikos, 2008). Pluronics have been widely used as an ocular drug delivery system because they could prolong drug release and present satisfactory inertia for eye tissue (Dumortier et al., 2006). However, a major disadvantage of Pluronics is their low mucoadhesive activity, therefore, some Pluronic-based ophthalmic formulations have been improved by adding polymers providing mucoadhesive property such as Carbopol (Qi et al., 2007), sodium hyaluronate (Wei et al., 2002).

Diclofenac sodium is one of non-steroidal anti-inflammatory drugs currently approved by US Food and Drug Administration for ocular use. It can suppress arachidonic acid transformation catalysed by cyclooxygenase enzymes leading to inhibition of prostaglandins synthesis in eyes (Araújo et al., 2009). Today, an ophthalmic preparation of diclofenac sodium available in the drug market is an eye drop dosage form only. However, it has been used extensively for the case of ophthalmic inflammatory with 3–4 times of administration. Thus, minimized frequency of administration, once or twice a day, is crucial to increase patient compliance. In this study, the formulations of diclofenac sodium for ophthalmic use would be formulated by using either Pluronic F127 or combination of Pluronic F127 and Pluronic F68 as a thermoresponsive gelling agent and also a solubility enhancer for diclofenac sodium instead of cyclodextrins which usually found in several marketed diclofenac sodium eye drops.

In manufacturing, ophthalmic solutions need to be sterilized to ensure sterility of the finished products. It can be made by using recommended methods either sterilization by filtration or autoclaving (steam sterilization) (Banker and Rhodes, 2002). Although autoclaving is the first choice for most ophthalmic solutions because of its convenience for large-scale production, some products could not be sterilized by this method since their physicochemical properties were altered under autoclaving conditions. Therefore, manufacturers must find the most appropriate method for sterilizing their ophthalmic products (Roy et al., 2001). Due to lack of evidence of suitable sterilization method for thermoresponsive diclofenac sodium ophthalmic in situ gels, this study would investigate whether autoclaving affected the physicochemical properties of them.

The objectives of this study were to optimize the formulation of thermoresponsive diclofenac sodium ophthalmic in situ gels (called briefly "DS in situ gels"), to evaluate their physicochemical properties, potential for being an eye irritant, in vivo ophthalmic absorption of diclofenac sodium, and to determine the effect of autoclaving on their physicochemical properties.

2. Materials and methods

2.1. Materials and animals

Pluronic F127 (F127) and Pluronic F68 (F68) (BASF) were purchased via The Sun Co., Ltd., Bangkok, Thailand. Carbopol 940 and diclofenac sodium (Sigma Aldrich) were supplied by Tong Chemical Co., Ltd., Bangkok, Thailand. All other chemicals and solvents used in this study were analytical grade and were obtained from Samchai Chemical Co., Ltd., Bangkok, Thailand.

Male and female New Zealand white rabbits weighing 2–2.5 kg were purchased from National Laboratory Animal Center, Mahidol University, Thailand. The procedure for use and care of animals for this study were approved by the Ethical Committee of Laboratory Animal Use of Rangsit University.

2.2. Methods

2.2.1. Preparation of DS in situ gels

The formulations of DS in situ gel containing various gelling agents were shown in Table 1. They were prepared on a weight basis by the cold method described by Wei et al. (2002). Briefly, F127 and F68 (in the cases of formulations containing F68) were completely dissolved in cold phosphate buffer pH 7.4 except for the formulations containing Carbopol 940, phosphate buffer pH 5.5 was used instead to avoid gel formation of Carbopol 940. Then, diclofenac sodium, benzalkonium chloride and Carbopol 940 (in the cases of formulations containing Carbopol 940) were respectively added into the solutions of gelling agents and stirred continuously until homogeneous solutions were obtained.

All samples were analysed spectrophotometrically in triplicate for the content uniformity at a wavelength of 276 nm (UV–Visible Spectrophotometer, Hitachi U-2000, Japan). Only samples with diclofenac sodium content within 100 ± 10% of labeled amount were accepted. The pH value of the preparations was determined in triplicate by using a pH meter (Hanna instruments 8417, USA).

2.2.2. Determination of flow ability of DS in situ gels

A test tube inverting method (Jeong et al., 2002) was employed to roughly determine the phase behavior of DS in situ gels at each temperature: 2 ± 1 °C (storage temperature), 27 ± 1 °C (average room temperature in Thailand) and 35 ± 1 °C (precorneal temperature). After turning down a test tube containing a sample, the sample was observed its flow. The samples flowing at 2 ± 1 °C and 27 ± 1 °C but not flowing at 35 ± 1 °C within 30 s were accepted as optimum thermoresponsive in situ gels for this study.

Table 2
Results of evaluation of thermoresponsive diclofenac sodium ophthalmic in situ gels.

Formulation	Transparency at temperatures (°C)[a]			pH	%LA	Flow ability at temperatures (°C)[b]			$T_{sol-gel}$ (°C)	Dissolution time of gels in STF (min)
	2 ± 1	27 ± 1	35 ± 1			2 ± 1	27 ± 1	35 ± 1		
F127-14	+++	+++	+++	7.4 ± 0.0	101.4 ± 0.6	+++	+++	++	38.6 ± 2.4	n.d.
F127-20	+++	+++	+++	7.4 ± 0.0	103.2 ± 1.0	+++	–	–	20.7 ± 0.1	n.d.
F127-26	+++	+++	+++	7.4 ± 0.1	102.1 ± 0.4	+++	–	–	14.4 ± 0.1	n.d.
F127-20 + F68-8	+++	+++	+++	7.3 ± 0.1	103.3 ± 1.2	+++	+	–	27.2 ± 0.3	n.d.
F127-20 + F68-11	+++	+++	+++	7.4 ± 0.1	102.6 ± 0.6	+++	+++	–	31.6 ± 1.8	20.2 ± 0.8
F127-20 + F68-14	+++	+++	+++	7.6 ± 0.1	101.2 ± 0.3	+++	++	–	34.3 ± 0.6	n.d.
F127-20 + F68-11 + C0.1	++	++	++	5.4 ± 0.1	103.2 ± 0.8	+++	++	–	32.6 ± 1.1	40.4 ± 0.7
F127-20 + F68-11 + C0.3	+	+	+	4.8 ± 0.1	103.3 ± 0.9	+++	++	–	31.7 ± 0.5	45.3 ± 0.6

(n = 3, mean ± SD), %LA = %labeled amount, $T_{sol-gel}$ = sol–gel transition temperature, n.d. = not determined.
[a] Transparency: +++ = transparent; ++ = slightly translucent; + = translucent; – = turbid.
[b] Flow ability: +++ = very good; ++ = good; + = average; – = not flow.

2.2.3. Gelling capacity test

Gelling capacity of the representative formulations was determined by placing a drop of the sample (about 20 μl) into a test tube containing 2 ml of pH 7.4 simulated tear fluid (STF) equilibrated at 35 ± 1 °C. The visual assessment of gel formation and dissolution with time record was performed in triplicate. The compositions of STF were sodium chloride 0.67 g, sodium bicarbonate 0.2 g, calcium chloride·$2H_2O$ 0.008 g, and purified water added to 100 g (Wu et al., 2007).

2.2.4. Rheological properties measurement of DS in situ gels

The rheological measurement was performed via a controlled stress rheometer equipped with a cone (0.8°) and plate geometry possessing diameter of 40 mm (Bohlin Gemini HR nano, Malvern instrument, UK). The samples were measured in triplicate by using the following tests:

2.2.4.1. Temperature sweep test. The temperature sweep test was performed to define the sol–gel transition temperature of DS in situ gels. The strain used in this study was fixed at 1% which was within the linear viscoelastic regime. Storage modulus (G') and loss modulus (G'') values of samples at varied temperatures (0–50 °C) were measured. The sol–gel transition temperature was defined as the medial temperature between those for the solution and the gel providing G' equaled G'' (Wei et al., 2002).

2.2.4.2. Steady shear sweep test. The steady shear sweep experiment was carried out for determination of flow behavior and viscosity of a representative of DS in situ gels. It was assessed in the mimicked physiological conditions where it was diluted with STF at a ratio of DS in situ gel to STF = 40:7 (35 ± 1 °C) and non-physiological conditions (27 ± 1 °C and 35 ± 1 °C without dilution by STF) (Qi et al., 2007). The initial and final shear rates were set at 0.05 and 100 s^{-1}, respectively.

2.2.5. Autoclaving sterilization

To study the effect of autoclaving sterilization on physicochemical properties of DS in situ gels, a group of representatives was treated under the autoclaving sterilization conditions following recommendation by the US Pharmacopeia 31 (The United States Pharmacopeial Convention, 2007). Briefly, screw cap test tubes containing 10 g of DS situ gel were placed in an autoclave (Hirayama, Japan). All of them were exposed to steam at 121 °C, under a pressure of about 15 psi, for 20 min. Then, they were evaluated their physicochemical properties i.e., flow ability, % labeled amount, pH, sol–gel transition temperature and flow behavior, and compared to those of them before being autoclaved.

2.2.6. Eye irritation test

2.2.6.1. In vitro eye irritation test. The in vitro eye irritation test was performed to screen the potential of DS situ gels for being an eye irritant before the in vivo test would be performed in rabbits. The protocol of this experiment followed the short time exposure (STE) test introduced by Takahashi et al. (2008). Briefly, SIRC (rabbit corneal cell line) cells purchased from American Type Culture Collection (USA) were cultured in Eagles's MEM (Invitrogen, USA) containing 10% (v/v) fetal bovine serum (Invitrogen, USA) and 2 mM L-glutamine (Invitrogen, USA) at 37 °C in 5% CO_2 atmosphere and subcultured every 3–4 days using trypsin–EDTA solution (Invitrogen, USA). The cells were seeded in 96-well plates with a density of 5.0×10^3 cells/well. After incubation for 5 days, the cells reached confluence and were exposed to 200 μl of either 5% or 0.05% of the representative diluted in normal saline for 5 min. After exposure, the cells were washed twice with phosphate buffer (Invitrogen, USA). Then, 200 μl of 0.5 mg/ml methylthiazolydiphenyl-tetrazolium bromide (MTT) (Sigma–Aldrich, USA) solution in medium was added and incubated for 2 h. The MTT formazan was extracted with 0.04 N HCl–isopropanol for 30 min and the absorbance of the extract was measured at 570 nm with a plate reader (Molecular Devices, USA). Wells containing medium and MTT solution without SIRC cells were used as a blank. The 100% cell viability was calculated from the results of wells containing SIRC cells with no exposure of the test solution. The assay was performed in 6-replicate and reported as mean %cell viability ± SD.

The potential for being an eye irritant from STE test were calculated as the followings: the 5% test concentration with %cell viability greater than 70% was scored 0, less than or equal 70% was scored 1; the 0.05% test concentration with %cell viability greater than 70% was scored 1, less than or equal 70% was scored 2. Then, the scores obtained from 5% test and 0.05% test were added together to give an eye irritation ranking. A rank of scored 1, 2 and 3 were categorized as a minimal ocular irritant, a moderate ocular irritant and a severe ocular irritant, respectively. If the sample was a minimal ocular irritant, it would be accepted for the in vivo eye irritant assessment.

2.2.6.2. In vivo eye irritation test. The in vivo eye irritation test of the representative of DS in situ gels was performed in a group of eight New Zealand albino rabbits. Twenty microliters of the representative formulation was instilled into the lower conjunctival sac of the rabbit's right eye, while the left was kept as a control without manipulation. The test eye was observed at each of the following time intervals: 0, 5, 10, 30 min, 1, 6, 12, 24, 48 and 72 h for the changes of cornea, iris, conjunctiva and chemosis compared to the control. The degree of eye irritation was scored following the modified Draize test (Bozdağ et al., 2008).

2.2.7. In vivo ophthalmic absorption of diclofenac sodium

Eight New Zealand albino rabbits were divided into 2 groups equally. Each group was treated with either the representative DS in situ gel or the commercial diclofenac sodium eye drop (0.1%, w/v) by instilling 20 μl of each test formulation into the conjunctiva sac of the rabbit's right eye. The rabbits were anesthetized with Zoletil 100® (Virbac, France) after instillation for 0, 5, 10, 30, 60, 360, and 720 min to withdraw 200 μl of aqueous humor from the treated eyes. Diclofenac sodium content in aqueous humor was determined by using a High Performance Liquid Chromatography (HPLC) technique (Riegel and Ellis, 1994). Briefly, the HPLC instrument (Agilent HP, USA) with a 20-μl auto-injection and a UV detector for UV detection at 280 nm was used in this study. The column (Agilent, USA) for separation was an ultrasphere reversed-phase octyl column (15 cm × 4.5 mm), with particle size of 5 μm. The mobile phase consisted of 505 ml acetonitrile containing 0.65 ml triethylamine, and 495 ml 1.65% glacial acetic acid and had an apparent pH of 4.35. It was pumped at a flow rate of 1.0 ml/min. The temperature was set at 30 °C. The samples were prepared as the followings: a 100-μl aliquot of the standard solution or sample was placed in a centrifuge tube containing 500 μl of acetonitrile. Naproxen used as an internal standard was added and the mixture was mixed mechanically for 90 s. The protein in aqueous humor was removed by centrifugation for 20 min at 60 rpm. The supernatant was transferred to a new tube, dried under nitrogen at room temperature and the residue was dissolved in 50 μl of mobile phase by swirl mixing for 1 min. Then, it was analysed for the content of diclofenac sodium using HPLC instrument and pharmacokinetic parameters of diclofenac sodium in the aqueous humors were calculated.

3. Results and discussion

3.1. Preparation of DS in situ gels

Most of DS in situ gels prepared by cold method for this study were transparent at all test temperatures (2 ± 1, 27 ± 1 and 35 ± 1 °C). This suggested that the ingredients in DS in situ gel formulations could be dissolved completely in the vehicle at all test temperatures due to the solubility enhancer effect of Pluronic (Dumortier et al., 2006). Except for the Carbopol 940-containing formulations, i.e., F127-20 + F68-11 + C0.1 and F127-20 + F68-11 + C0.3, they were slightly translucent and translucent, respectively. This might be that pH of these formulations (Table 2) were lower than p*K*a of Carbopol which is 6 ± 0.5 (Wu et al., 2007) leading to incompletely swelling of Carbopol (Piau, 2007). It is pertinent to note that although pH 5.5 phosphate buffer was used as vehicle for F127-20 + F68-11 + C0.1 and F127-20 + F68-11 + C0.3, their pH still tended to decrease with the increment of Carbopol concentration. This might be due to the more acidity from acrylic acid units of Carbopol when the more Carbopol was added (Piau, 2007) and moreover, the low buffer capacity of 0.02 M phosphate buffer (pH 5.5).

The results of content uniformity analysis of DS in situ gels presented as %labeled amount shown in Table 2 suggested that all products could be accepted for the further experiments because they contained diclofenac sodium within the range of 100 ± 10% of labeled amount.

3.2. Determination of flow ability and sol–gel transition temperature of DS in situ gels

The results of determination of flow ability and sol–gel transition temperature of DS in situ gels were shown in Table 2 and discussed separately according to the formulation compositions which might influence flow ability and sol–gel transition.

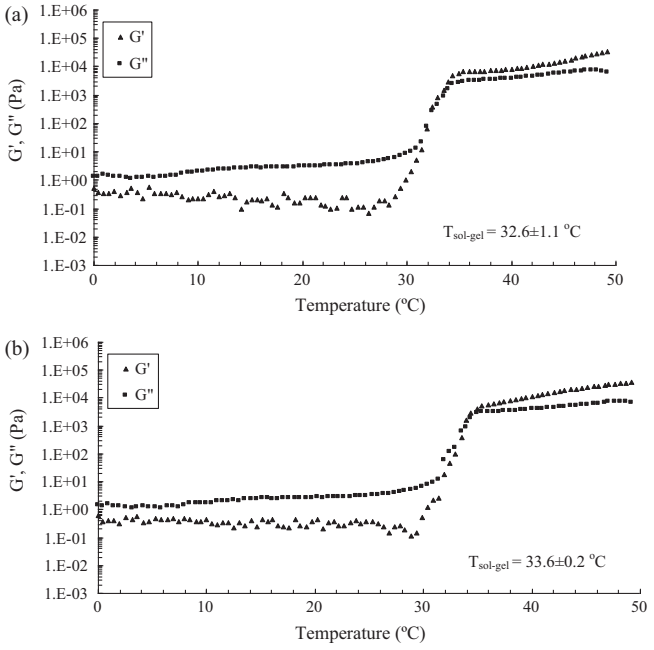

Fig. 1. Semi-logarithmic plot of G' and G'' against temperatures of F127-20 + F68-11 + C0.1, (a): before autoclaving and (b): after autoclaving.

To determine the sol–gel transition temperature of DS in situ gels, the semi-logarithmic plots of G' and G'' against temperatures obtained from the temperature sweep test were constructed and shown in Fig. 1 as a representative. It was defined as the average temperature providing G' equaled to G''.

3.2.1. Effect of F127 concentration

Table 2 indicated that F127-14 containing the lowest concentration of F127 could flow freely like water at all test temperatures. This suggested that F127-14 could not exhibit gel characteristic at any test temperatures. However, the products containing more concentration of F127 i.e., F127-20 and F127-26 could flow freely at 2 ± 1 °C but became stiff gel at 27 ± 1 and 35 ± 1 °C. This finding was consistent with the results of determination of sol–gel transition temperature by using the temperature sweep test, which indicated that the sol–gel transition temperature of F127-14 was 38.6 ± 2.4 °C, higher than the maximum test temperature. Therefore, gel characteristic of F127-14 could not be observed by the test tube inverting method. On the other hand, the sol–gel transition temperature of F127-20 and F127-26 were 20.7 ± 0.1 °C and 14.4 ± 0.1 °C, respectively, leading to the stiff gels at the temperature of 27 ± 1 °C and 35 ± 1 °C in the test tube inverting test.

The ranking of sol–gel transition temperature of the products using only F127 as a gelling agent was F127-14 > F127-20 > F127-26 implying that their sol–gel transition temperatures tended to decrease when the concentration of F127 was increased. This finding was according to the previous work reported by Vadnere et al. (1984) and could be explained by the changes in properties of PEO and PPO domains consisting in F127 molecules after the changes in either concentration or temperature of the Pluronic solution system (Klouda and Mikos, 2008). At the temperature below the critical micelle temperature, both PEO and PPO in Pluronic molecules were hydrated. When temperature increased, PPO chains became being lesser soluble than PEO and dehydrated. This led to hydrophobic interactions among the PPO domains and formation of spherical micelles consisting of a dehydrated PPO core with an outer shell of hydrated swollen PEO. Then, the micelles would hardly occupy a high fraction volume of solution, and come into contact and entangle with each other resulting in a three-dimension network

Table 3
Effect of autoclaving sterilization on physicochemical properties of thermoresponsive diclofenac sodium ophthalmic in situ gels.

Formulations	Before autoclaving						After autoclaving					
	Flow ability at temperatures (°C)[a]			pH	%LA	$T_{sol-gel}$ (°C)	Flow ability at temperatures (°C)[a]			pH	%LA	$T_{sol-gel}$ (°C)
	2±1	27±1	35±1				2±1	27±1	35±1			
F127-20 (II)	+++	–	–	6.29 ± 0.01	96.1 ± 1.0	20.8 ± 0.2	+++	–	–	6.15 ± 0.01	90.2 ± 2.2	20.8 ± 0.3
F127-20 + F68-11 (II)	+++	+++	–	6.43 ± 0.01	98.4 ± 1.5	32.4 ± 1.3	+++	+++	–	6.36 ± 0.01	90.5 ± 1.3	32.9 ± 0.3
F127-20 + F68-11 + C0.1	+++	++	–	5.40 ± 0.02	98.8 ± 0.3	32.6 ± 1.1	+++	++	–	5.36 ± 0.01	92.8 ± 1.4	33.6 ± 0.2

(n = 3, mean± SD), %LA = %labeled amount, $T_{sol-gel}$ = sol–gel transition temperature.
[a] Flow ability: +++ = very good; ++ = good; + = average; – = not flow.

structure and forming stiff gel (Dumortier et al., 2006; Klouda and Mikos, 2008). Therefore, the products containing more effective concentration of F127 would contain more number of micelles. They would need lower energy to promote sol–gel transition and could perform sol–gel transition at lower temperature than products containing less F127 content.

3.2.2. Effect of F68 concentration

To optimize DS in situ gel formulations possessing optimum sol–gel transition temperature, F127-20 was selected as a prototype because it contained adequate amount of polymer and had a sol–gel transition temperature close to room temperature. In this study, F68, the more hydrophilic block co-polymer, was used as an auxiliary gelling agent for modification of the sol–gel transition temperature.

Table 2 shows that addition of F68 into F127-20 could modify flow ability and raise sol–gel transition temperature of DS in situ gels. F127-20 + F68-8, F127-20 + F68-11 and F127-20 + F68-14 could flow freely like water at 2 ± 1 °C and became stiff gel at 35 ± 1 °C. At 27 ± 1 °C, although F127-20 + F68-11 still could flow freely, F127-20 + F68-14 flowed more slowly and F127-20 + F68-8 was almost fixed. The sol–gel transition temperature of F127-20 + F68-8, F127-20 + F68-11 and F127-20 + F68-14 were 27.2 ± 0.3 °C, 31.6 ± 1.8 °C and 34.3 ± 0.6 °C, respectively. Consequently, it was reasonable to find that F127-20 + F68-8 was difficult to flow at 27 ± 1 °C. For the case of F127-20 + F68-11 and F127-20 + F68-14, the transition temperature were more above 27 ± 1 °C. Both formulations were thus able to flow. However, F127-20 + F68-14 which contained more gelling agents exhibited highly viscous characteristic.

The comparison of sol–gel transition temperature of F127-20 + F68-8, F127-20 + F68-11 and F127-20 + F68-14 to that of F127-20 suggested that the products contained more F68 content would exhibit sol–gel transition at higher temperature. This might be that the more hydrophilic F68 consisting of lower ratio of PPO units/PEO units per mole, 0.19, compared to F127, 0.32, could disrupt the hydration shells around the hydrophobic portion of F127 molecules. It caused more local high order of water molecules around the hydrophobic PPO units. When gelling process occurred, these ordered water molecules had to be squeezed out into the bulk solution. Therefore, the increase in temperature was needed for the system to promote the hydrophobic interaction between Pluronic micelles (Vadnere et al., 1984). However, Wei et al. (2002) reported that addition of too high F68 concentration into F127 based thermoresponsive gels might decrease sol–gel transition temperature of the products due to increase in effective concentration of F68. Therefore, the optimum of F68 concentration should be considered for optimization of F127-based ophthalmic in situ gel formulations.

Although F127-20 + F68-11 could transform to be stiff gel at lower temperature than that of F127-20 + F68-14, it was chosen for the further study because it contained lower F68 concentration and exhibited more sol characteristic than that of F127-20 + F68-14 at room temperature. Therefore, it could be dropped into the eye easier than that of F127-20 + F68-14.

3.2.3. Effect of Carbopol 940 concentration

Table 2 points out that addition of Carbopol 940 into F127-20 + F68-11 at concentration of 0.1% and 0.3% could slightly alter flow ability by increase in viscosity. But it could not alter sol–gel transition temperature of the products significantly. The comparison of sol–gel transition temperature of F127-20 + F68-11 + C0.1 and F127-20 + F68-11 + C0.3 to that of F127-20 + F68-11 by using one way ANOVA with Tukey HSD showed that they were comparable at p-value > 0.05. Nevertheless, this finding did not correspond to the previous work by Ryu et al. (1999) reporting that Carbopol tended to decrease sol–gel transition of Pluroinc based suppository. This might be that the concentrations of Carbopol used in present study were quite low to avoid highly viscous, turbid and acidic gels, thus, the effect of Carbopol on sol–gel transition temperature of Pluronic-based in situ gel could not be found obviously. Due to possessing optimum sol–gel transition temperature, F127-20 + F68-11, F127-20 + F68-11 + C0.1 and F127-20 + F68-11 + C0.3 were used for the gelling capacity test.

3.3. Gelling capacity test

The results of gelling capacity test of the representatives i.e., F127-20 + F68-11, F127-20 + F68-11 + C0.1 and F127-20 + F68-11 + C0.3, were presented as dissolution time of the gel in STF (pH 7.4) in Table 2. Although all of them could form gel immediately when they reached the STF equilibrated at 35 ± 1 °C, the formulations containing Carbopol 940 (F127-20 + F68-11 + C0.1 and F127-20 + F68-11 + C0.3) were eroded by STF about 2 times slower than the formulation without Carbopol 940 (F127-20 + F68-11). However, F127-20 + F68-11 + C0.3 which contained high Carbopol 940 content could more tolerate erosion by STF than that of F127-20 + F68-11 + C0.1. This is probably that STF (pH 7.4) led to ionization of carboxyl groups in Carbopol molecules and thus repulsion of these ions. Then, Carbopol would be in an extended configuration and form strong three-dimensional networks (Piau, 2007). Therefore, the formulation contained more Carbopol content would possess stronger gel structure. However, since F127-20 + F68-11 + C0.1 not only contained the lowest content of acidic Carbopol but also had optimum sol–gel transition temperature and physicochemical properties such as pH, it was accepted as an optimum formulation and selected for the more evaluations.

3.4. Steady shear sweep test

The flow behavior and viscosity profile of F127-20 + F68-11 + C0.1 were investigated using steady shear sweep test at room temperature (27 ± 1 °C) and at precorneal temperature (35 ± 1 °C) with either dilution or non-dilution by STF. Rheograms and vis-

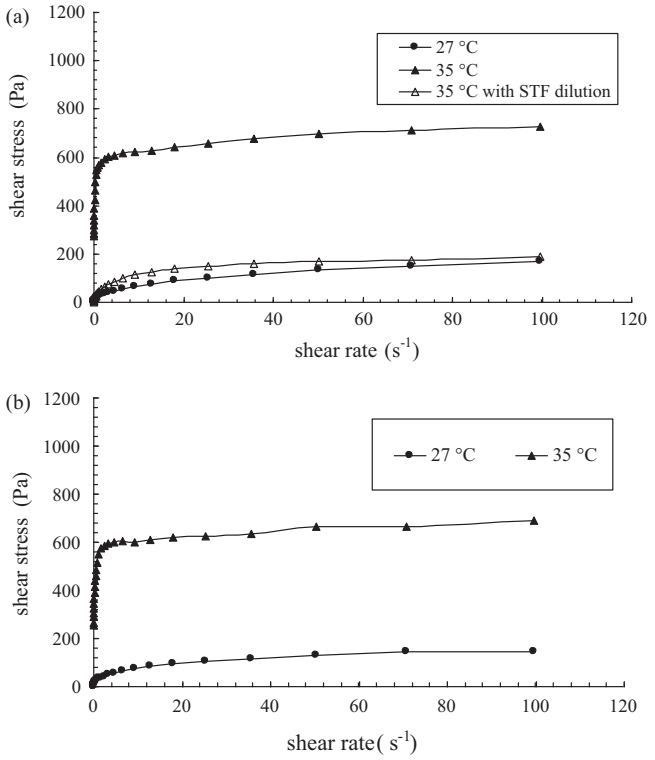

Fig. 2. Rheogram of F127-20 + F68-11 + C0.1, (a): before autoclaving and (b): after autoclaving.

cosity profiles obtained from this test shown in Fig. 2(a) and Fig. 3, respectively, indicating that F127-20 + F68-11 + C0.1 exhibited pseudoplastic flow at all test conditions. This suggested that the three-dimension network of F127-20 + F68-11 + C0.1 was gradually destroyed by increased shear rate (Sinko, 2006), however, it led to an advantage of F127-20 + F68-11 + C0.1. Generally, the ocular shear rate during interblinking is quite low, about 0.03 s^{-1}, and becomes higher during blinking, about 4250–28,000 s^{-1} (Srividya et al., 2001). Therefore, both undiluted and diluted F127-20 + F68-11 + C0.1 (at 35 ± 1 °C) possessing high viscosity under the low shear rate and low viscosity under the high shear rate could prolong contact time and could not cause eye irritation after instillation. In addition, at the temperature of 27 ± 1 °C, F127-20 + F68-11 + C0.1 had low viscosity, consequently, it could be instilled into the eye easily without keeping in a fridge.

Although the viscosity of F127-20 + F68-11 + C0.1 at 35 ± 1 °C was markedly decreased when it was diluted with STF, its viscosity was still higher than that of low viscous F127-20 + F68-11 + C0.1 at 27 ± 1 °C. This indicated that F127-20 + F68-11 + C0.1 has a potential for increasing contact time of diclofenac sodium in the eye.

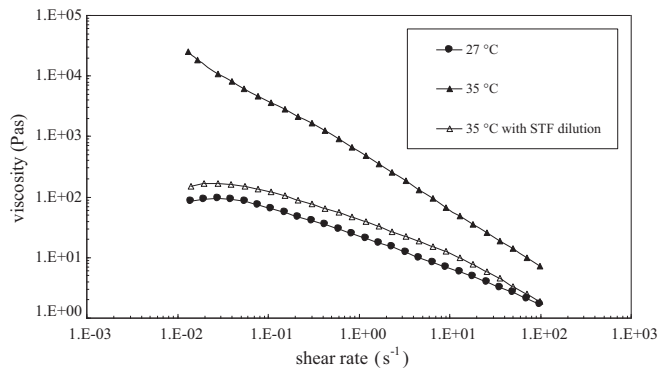

Fig. 3. Logarithmic plot of viscosity against shear rate of F127-20 + F68-11 + C0.1 (without autoclaving).

3.5. Autoclaving sterilization

The effect of autoclaving sterilization on physicochemical properties of F127-20 + F68-11 + C0.1 was determined. In addition, to obtain more information, F127-20 and F127-20 + F68-11 using the same vehicle as F127-20 + F68-11 + C0.1, pH 5.5 phosphate buffer, instead of pH 7.4 phosphate buffer were also examined and, for this part, they were called F127-20 (II) and F127-20 + F68-11 (II), respectively. It was found that autoclaving sterilization could not alter the flow ability, the sol–gel transition temperature and also the flow behavior of all test samples significantly as seen in Table 3, Figs. 1(b) and 2(b). This suggested that autoclaving sterilization could not change the physicochemical properties of gelling agents significantly. This finding agreed with the previous report (Dumortier et al., 2006) indicating that sterilization by autoclaving did not significantly alter rheological characteristic of Pluronic F127-based formulations. However, the Table 3 shows that the %labeled amount and the pH of all formulations were significantly decreased after they had been autoclaved (p-value = 0.001 and 0.000, respectively, analysed by using Paired sample T-test). This pointed out that autoclaving could significantly induce chemical changes of the compositions consisting in the test samples. Due to significant decrease in the %labeled amount and the pH of all test samples, the major change might be from chemical degradation of diclofenac sodium. This was consistent with Roy et al. (2001) reporting that autoclaving could accelerate chemical degradation of diclofenac sodium in the diclofenac sodium injection and produce impurities. Nevertheless, Swamy et al. (2010) reported that diclofenac sodium gels using sodium carboxymethyl hydroxypropyl guar as a gelling agent stored at 25 °C, 60% RH and 40 °C, 70% RH were chemically and physically stable throughout the 6-month experiment. Consequently, to meet the criteria of sterility and stability for the ophthalmic products, a manufacturing process avoiding extremely high temperature such as using aseptic technique with sterilization by filtration at room temperature should be considered as a more appropriate method for manufacture of DS in situ gels than the process with terminal sterilization by autoclaving.

However, since the condition of the previous works performed by Roy et al. (2001) and Swamy et al. (2010) were different from this present study, the degradation of diclofenac sodium consisting in DS in situ gels during autoclaving and long-term stability of DS in situ gels manufactured without excess heat should be investigated further to understand the mechanism of this chemical degradation.

3.6. Eye irritation test

The in vitro eye irritation test of F127-20 + F68-11 + C0.1 performed in SIRC cells following the STE test showed that the SIRC cells could tolerate the 5% and the 0.05% of F127-20 + F68-11 + C0.1 diluted in medium very well. The percentage cell viability (%cell viability) of SIRC cells exposed to the 5% and the 0.05% F127-20 + F68-11 + C0.1 were 95.5 ± 3.9% (scored 0) and 98.1 ± 1.9% (scored 1), respectively. The total score of potential for being an eye irritant of F127-20 + F68-11 + C0.1 was 1. Consequently, F127-20 + F68-11 + C0.1 was classified as a minimal ocular irritant and safe to test in rabbits.

The result of eye irritation test of F127-20 + F68-11 + C0.1 studied in rabbits following modified Draize test was shown in Table 4. It indicated that F127-20 + F68-11 + C0.1 did not irritate rabbits' eyes as seen in the total score of an eye irritation assessment equaling to 0. Therefore, F127-20 + F68-11 + C0.1 could be accepted as safe for ophthalmic use.

Table 4
Score obtained from eye irritation assessment of F127-20 + F68-11 + C0.1 in rabbits.

Lesion	Score for each lesion	Score obtained from assessment
A-Conjuctival edema (chemosis)		
No swelling	0	0
Any swelling	1	
Prominent swelling along with partial lid eversion	2	
Swelling with half-closed lids	3	
Swelling with totally closed lids	4	
B-Redness in conjunctiva		
Absent	0	0
Abnormal conjunctival injections	1	
More diffuse and deeper hyperaemia, separate vessels can not be seen easily	2	
Diffuse and dense hyperaemia	3	
C-Secretion		
Absent	0	0
Any abnormal secretion	1	
Secretion leading to wet eye lashes closer to lids	2	
Secretion leading to wet lids and whole periorbital area	3	
D-Corneal opacity		
Absent	0	0
Scattered or diffused areas-detail of the iris discernible	1	
Easy discernable, transparent areas, detail of the iris slightly darkened	2	
Opalescent areas, no details of the iris discernible, size of the pupil barely discernible	3	
Opaque cornea, iris not discernible	4	
E-Iris involvement		
Absent	0	0
Pronounced deep folds, congestion, deep swelling, circumcorneal injection, the iris still reacts to light	1	
No response, haemorrhage, marked destruction	2	
	Total score	0

[adapted from Bozdağ et al. (2008)].

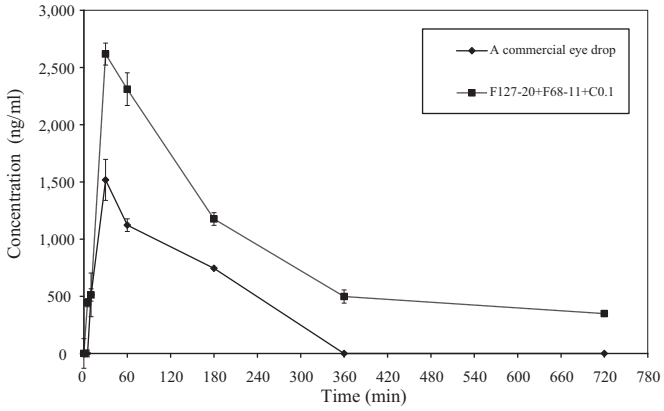

Fig. 4. Concentration of diclofenac sodium in rabbits' aqueous humor at various times after instillation of a commercial diclofenac sodium eye drop and F127-20 + F68-11 + C0.1 ($n = 4$, mean ± SD).

3.7. In vivo ophthalmic absorption of diclofenac sodium

Fig. 4 shows the concentration of diclofenac sodium in rabbits' aqueous humor at various times after instillation of 20 μl of a commercial diclofenac sodium eye drop and F127-20 + F68-11 + C0.1 into the rabbits' eyes. The pharmacokinetic parameters of diclofenac sodium in aqueous humor after instillation of these formulations were shown in Table 5 and statistically compared by using the Paired sample *T*-test. It was found that the maximum concentration (C_{max}) of diclofenac sodium in rabbits' aqueous humor after instillation of F127-20 + F68-11 + C0.1 was 2.2-fold higher than that obtained after instillation of the commercial eye drop (p-value = 0.000). In comparison with the commercial eye drop, F127-20 + F68-11 + C0.1 showed 1.7-fold and 5.6-fold greater the time to reach C_{max} of diclofenac sodium in the aqueous humor (T_{max}) and the content of diclofenac sodium absorbed into the aqueous humor entire this experiment calculated as the area under the curve (AUC), respectively (p-value = 0.000 and 0.000). The comparison of constant of absorption rates (K_a), $t_{1/2}$ (K_a), the constant of elimination rates (K_e), and $t_{1/2}$ (K_a) suggested that diclofenac sodium from the commercial eye drop was absorbed into the aqueous humor and then eliminated from the aqueous humor more quickly than that of F127-20 + F68-11 + C0.1 (p-value = 0.000, 0.000, 0.000 and 0.001, respectively). Consequently, the diclofenac sodium content could not be found in rabbits' aqueous humor at 360 and 720 min after instillation of the commercial eye drop. These suggested that F127-20 + F68-11 + C0.1 could increase the diclofenac sodium content absorbed into the eyes and prolong the elimination time of diclofenac sodium from aqueous humor leading to the better ophthalmic bioavailability of diclofenac sodium than that of the commercial diclofenac sodium eye drop.

Moreover, at 720 min after instillation of the DS in situ gel into the eyes, the concentration of diclofenac sodium in aqueous

Table 5
Pharmacokinetic parameters of diclofenac sodium in rabbits' aqueous humor.

Pharmacokinetic parameters	A commercial eye drop	F127-20 + F68-11 + C0.1
C_{max} (ng/ml)	1154.7 ± 98.9	2584.4 ± 136.7
T_{max} (min)	12.5 ± 0.1	21.1 ± 0.8
$AUC_{0-720\,min}$ (ng × min/ml)	$1.5 \times 10^5 \pm 8.3 \times 10^3$	$8.4 \times 10^5 \pm 3.7 \times 10^4$
K_a (1/min)	0.3 ± 0.0	0.2 ± 0.0
K_e (1/min)	$1.2 \times 10^{-2} \pm 2 \times 10^{-4}$	$0.3 \times 10^{-2} \pm 3 \times 10^{-4}$
$t_{1/2}$ (K_a) (min)	2.7 ± 0.0	3.5 ± 0.1
$t_{1/2}$ (K_e) (min)	59.8 ± 0.9	211.3 ± 18.9

humor was 349.0 ± 57.7 ng/ml. It was higher than the upper value of in vivo minimum effective concentration (MEC) of diclofenac sodium which was in the range of 14–158.2 ng/ml (Nishihata et al., 1988) about 2.2 folds. This suggested that the DS in situ gel could maintain the concentration of diclofenac sodium in aqueous humor above MEC at least 12 h after instillation. On the other hand, the aqueous humor obtained from the eyes treated with the commercial diclofenac sodium eye drop could not be determined for the diclofenac sodium content since 360 min. This pointed out that at 6 h, the diclofenac sodium content was lower than the limit of detection of the analysis (10 ng/ml) and the lower value of MEC. Therefore, to maintain the diclofenac sodium concentration in aqueous humor above MEC, F127-20 + F68-11 + C0.1 could be administered with lower frequency than the commercial diclofenac sodium eye drop.

4. Conclusions

The physicochemical properties of F127-based DS in situ gels were affected by compositions in formulation. It was found that increment of F127 content could decrease sol–gel transition temperature of the products. On the other hand, increase in F68 concentration within the optimum range would increase sol–gel transition temperature. Although sol–gel transition temperature of DS in situ gels was not significantly altered by the low concentration of Carbopol 940 (0.1–0.3%, w/w), transparency, pH, and gelling capacity of the products would be changed obviously by it. The optimized DS in situ gel formulation obtained from this study was F127-20 + F68-11 + C0.1 containing 20% (w/w) F127, 11% (w/w) F68, and 0.1% (w/w) Carbopol 940 as gelling agents. It could be dropped into the eyes at the room temperature easily and performed strong gel with pseudoplastic flow behavior at the precorneal temperature. Nevertheless, the study of effect of autoclaving sterilization on physicochemical properties of F127-20 + F68-11 + C0.1 suggested that autoclaving could not be considered as a suitable method for sterilization of this product because it could lead to chemical degradation of diclofenac sodium even though its sol–gel transition temperature had not been changed. In vitro and in vivo eye irritation test of F127-20 + F68-11 + C0.1 showed that it could be accepted as safe for ophthalmic use. In addition, in vivo evaluation of ophthalmic absorption indicated that F127-20 + F68-11 + C0.1 could increase ophthalmic bioavailability of diclofenac sodium in rabbits and reduce the frequency of administration significantly compared to a commercial product. Therefore, the optimized formulation of thermoresponsive diclofenac sodium ophthalmic in situ gel developed in this study had potential for using as an alternative to conventional diclofenac sodium eye drop to increase patient compliance. However, the suitable sterilization method for this product should be the method avoiding excess heat such as sterilization by filtration at room temperature.

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