International Journal of Pharmaceutics 410 (2011) 31–40

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Design and evaluation of baicalin-containing in situ pH-triggered gelling system for sustained ophthalmic drug delivery

Haoyun Wu[a,b,c], Zhidong Liu[a,b,c,*], Junjie Peng[d], Lin Li[a,b,c], Nan Li[a,b,c], Jiawei Li[a,b,c], Hao Pan[d]

[a] Engineering Research Center of Modern Chinese Medicine Discovery and Preparation Technique, Ministry of Education, 88 Yuquan Road, Tianjin 300193, PR China
[b] Institute of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine, 88 Yuquan Road, Tianjin 300193, PR China
[c] Tianjin State Key Laboratory of Modern Chinese Medicine, 88 Yuquan Road, Tianjin 300193, PR China
[d] School of Pharmacy, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, PR China

ARTICLE INFO

Article history:
Received 26 January 2011
Accepted 7 March 2011
Available online 11 March 2011

Keywords:
Sustained ophthalmic delivery
In situ gelling system
Baicalin
Carbopol
Hydroxypropylmethylcellulose

ABSTRACT

Baicalin has been reported to have anti-inflammatory and anti-cataract effects on eye tissues, but it has a low bioavailability partly due to its poor stability of baicalin, the special anatomic structure and efficient protective mechanism of eyes. The aim of this study was to investigate the correlation between the stability of baicalin and in situ pH-triggered gelling system. Carbopol® 974P (0.3%, w/v) was used as the gelling agent combined with hydroxypropylmethylcellulose E4M (0.6%, w/v) which acted as a viscosity enhancing agent. In vitro and in vivo evaluations were performed using several techniques, namely confocal scanning light microscopy analysis, rheometry, Gamma scintigraphic technique and microdialysis method. The rheological behavior showed a significant enhancement in gel strength under physiological conditions, and the formulation provided sustained release of the drug over an 8-h period. In elimination studies, the radioactivity of formulation was always higher than that of the control solution. Additionally, the AUC and C_{max} values were 6.1-fold and 3.6-fold higher than those of the control solution, respectively. The results demonstrated that an in situ pH-triggered gelling system have better ability to keep baicalin stable and retain drug release than marketed baicalin eye drops to enhance the ocular bioavailability.

1. Introduction

Baicalin (β-D-gluopyranosiduronic acid, 5, 6-dihydroxy-4-oxo-2-phenyl-4H-1-benzopyran-7-yl) is a flavonoid purified from the medicinal plant *Scutellaria baicalensis* Georgi, which has been used in traditional Chinese medicine for thousands of years and is officially listed in the Chinese Pharmacopoeia. It has many significant biological effects on eye tissues, such as anti-inflammatory, anti-Chlamydia, antibacterial, anti-oxidative and anti-cataract (Cheng et al., 2001; Jung et al., 2008; Huang et al., 2009; Nakamura et al., 2003; Qi et al., 1998; Robinson and Mlynek, 1995). However, baicalin is practically insoluble in water and not stable in basic solution, and its solubility and stability are very sensitive to pH changes. Therefore, there are several limitations to making baicalin into proper ophthalmic preparation and achieve its therapeutic efficacy for eye diseases.

For the ophthalmic drug delivery, it is a challenging task due to normal ocular protective mechanisms such as blinking and tears drainage that promotes rapid clearance and reduces bioavailability resulting in a short duration of pharmacological response (Maurice, 1987). Only 1–10% of topically applied drug is absorbed (Nanjawade et al., 2007), which also includes absorption into the gastrointestinal tract due to drainage through the nasal–lacrimal duct (Sieg and Robinson, 1977). Frequent instillations of eye drops are necessary to maintain a therapeutic drug level in the tear film or at the site of action. But the frequent use of highly concentrated solutions may induce toxic side effects and cellular damage at the ocular surface (Baudouin, 1996; Salminen, 1990; Topalkara et al., 2000).

Current research efforts are focused on the design and evaluation of ophthalmic drug delivery systems that are easy to administer, require decreased administration frequency, and provide controlled and possibly sustained drug release in order to increase therapeutic efficacy and patient compliance. In situ pH-triggered gelling system are liquid upon instillation and undergo a phase transition to form a viscoelastic gel in response to pH changes, which are expected to provide prolonged corneal contact time, reduced precorneal drug loss, and provide convenience in administration as compared to eye drops, suspensions or ointments. Baicalin is most stable at the weak acidity solution of in situ

* Corresponding author at: Tianjin University of Traditional Chinese Medicine, 88 Yuquan Road, Tianjin 300193, PR China. Tel.: +86 222 305 1965; fax: +86 242 305 0129.
E-mail addresses: whycloudy@163.com (H. Wu), lonerliuzd@163.com (Z. Liu).

0378-5173/$ – see front matter.
doi:10.1016/j.ijpharm.2011.03.007

pH-triggered gelling system under non-physiological conditions. Therefore, it is necessary to design ophthalmic drug delivery system of baicalin that dose not only alleviate the shortcomings of conventional delivery vehicles, but also maintains baicalin stability and enhance its bioavailability at the property preparation.

The overall objective of the present work was to investigate the correlation between the stability of baicalin and in situ pH-triggered gelling system. To achieve this objective, in vitro and in vivo evaluations were comprehensively performed through the microstructure of gelling system, gelling capability studies, the rheological properties studies, in vivo elimination studies and pharmacokinetic studies.

2. Materials and methods

2.1. Materials and animals

2.1.1. Materials

Baicalin was purchased from ZhongXin Pharmaceutical (>98%, Tianjin, China). Carbopol (Carbopol® 974P NF) was kindly gifted by BF Goodrich (USA). HPMC (Methocel E4M) was obtained from Colorcon (UK). FITC (Conjugation grade) was provided by Sigma (USA). All other regents were of analytical grade.

2.1.2. Animals

New Zealand white rabbits, with weights between 2.5 and 3.0 kg, were provided by the Chinese Academy of Medical Sciences of Radiation Research Institute (License No.: SCXK JIN 2005-0001). The animals, housed in standard cages in a light-controlled room at (19 ± 1) °C and (50 ± 5)% R.H., were given a standard pellet diet and water ad libitum. All studies were conducted in accordance with the Principles of Laboratory Animal Care (NIH publication no. 92-93, revised in 1985) and were approved by the Department of Laboratory Animal Research at Tianjin University of Traditional Chinese Medicine. The procedures involving animals were reviewed and approved by the Animal Ethical Committee at Tianjin University of Traditional Chinese Medicine.

2.2. Preparation of formulations

2.2.1. The stability and solubility of baicalin test

Stability test of baicalin in different pH buffers: Baicalin was tested in distilled water and phosphate buffer USP (pH 5.8, 6.0, 6.2, 6.4, 6.6, 6.8 and 7.0). And the samples were withdrawn at 0, 0.5, 1.0, 1.5, 2.0, 4.0, 8.0 and 10.0 h. Then 20 μL of sample solution was injected for HPLC determination (Shimadzu LC 10-AT, Japan).

The solubility of baicalin test: Excess amount of baicalin was added into distilled water and phosphate buffer USP (pH 5.8 and 6.8), which were maintained at (35 ± 1) °C for 24 h using a magnetic stirrer (SCZL-4B, Henan, China) at a rotating speed of 200 rpm min^{-1}, to make sure excess baicalin always remain. After 24 h, the samples were centrifuged for 10 min at 4000 rpm min^{-1}. The collected supernatant was filtered with 0.45 μm microspore filter and the filtrate was diluted with distilled water and phosphate buffer USP (pH 5.8 and 6.8). Then 20 μL of sample solution was injected for HPLC determination (Shimadzu LC 10-AT, Japan).

2.2.2. Preparation of in situ gelling system

0.1% (w/v) baicalin, the required amount of the marketed eye drops, was firstly dissolved in 100 mL distilled water and dispersed well with ultrasonic cleaner (C3860A, Tianjin, China). And a certain amount of polymers (Carbopol and HPMC) were then slowly added to the baicalin solution with continuous magnetic stirring (SCZL-4B, Henan, China) for 2 h. All the gelling systems were adjusted to pH 5.8 by 0.5 M sodium hydroxide solution. Then the in situ pH-triggered gelling systems were obtained.

2.3. Formula optimization and drug release mechanism studies

2.3.1. Gelling capacity studies

Gelling systems of various concentrations of Carbopol and HPMC were prepared and evaluated for gelling capacity in order to identify the compositions suitable for use as an in situ gelling system. The gelling capacity was determined by placing 100 μL of in situ gelling system in a vial containing 2 mL of phosphate buffer at pH 6.8 and equilibrated at (35 ± 1) °C and visually assessing the gel formation and noting the time for gelation and the time taken for the gel formed to dissolve (Srividya et al., 2001).

2.3.2. In vitro release studies

In order to study drug release behavior of in situ pH-triggered gelling system, the in vitro release test was done using a membraneless dissolution model (Lin and Sung, 2000) with a dissolution testing apparatus (ZRS-8G, Tianjin, China). Gelling systems (3 g) of various concentrations were transferred into small vials (1.24 cm i.d. and 3.83 cm in depth) in triplicate and each vial was immersed into 1000 mL phosphate buffer at pH 6.8, which was used as the release medium. Care was taken to avoid the breaking of gelling system and the gel contained no bubbles. The temperature and stirring rate were maintained at (35 ± 1) °C and 75 rpm, respectively. At each time interval, 5 mL of aliquots were withdraw from the release mediums and replaced by an equal volume of release medium. The release of baicalin was analyzed by HPLC determination (Shimadzu LC 10-AT, Japan).

2.4. In vitro evaluation of formulations

2.4.1. Micro-morphology of in situ gelling system

The micro-morphology of in situ gelling system was observed under a confocal scanning light microscope (Zeiss LSM 710, Germany). FITC, as the fluorescent marker, was added into gelling system under non-physiological condition (25 ± 1 °C, pH 5.8) and physiological condition (35 ± 1 °C, pH 6.8), respectively. And then the FITC-labeled samples were instilled on glass slide and observed. The excitation wavelength of FITC was 488 nm, and emission maxima were between 490 and 540 nm (Tromp et al., 2001). Digital image files were acquired in multiple .tif format and in 1024 × 1024 pixel resolution.

2.4.2. Rheological studies

The rheological properties were determined using the small sample adaptor of the rheometer (Brookfield DV-III, USA). The viscosity of the different gelling systems was measured under non-physiological condition (25 ± 1 °C, pH 5.8) and physiological condition (35 ± 1 °C, pH 6.8), respectively. The temperature was maintained within ±1 °C by a recirculating bath (Wisdom) connected to the sample adaptor of the rheometer. The pH values of samples were raised to 6.8 by adding 0.5 M sodium hydroxide. The samples were equilibrated on the plate for 5 min to reach the running temperature prior to each measurement. A typical run comprised of changing the shear rate from 0 to 130 s^{-1} at a controlled ramp speed, a 0.1 min wait at 130 s^{-1}, and finally a decrease in shear rate to 0 s^{-1} at the same controlled ramp speed. Evaluations were conducted in triplicate.

2.4.3. Accelerated stability studies

Selected formulation F1-3 was stored at (4 ± 1) °C, room temperature (25 ± 1) °C, and (45 ± 1) °C for a period of 3 months. The formulations were evaluated at periodic for drug content (HPLC), pH, gelling capacity and in vitro drug release (Srividya et al., 2001).

Table 1
Stability of baicalin in different pH phosphate buffer solutions (mean ± SD, $n = 3$).

Medium	Concentration (%, w/v)							
	0 h	0.5 h	1.0 h	2.0 h	4.0 h	6.0 h	8.0 h	10 h
H_2O	100.0 ± 0.0	100.2 ± 0.2	100.2 ± 0.3	100.1 ± 0.2	100.0 ± 0.3	99.8 ± 0.2	100.1 ± 0.1	99.6 ± 0.2
pH 5.8 PBS	100.0 ± 0.0	100.5 ± 0.1	100.1 ± 0.2	100.2 ± 0.2	99.9 ± 0.2	100.0 ± 0.1	99.8 ± 0.2	99.8 ± 0.1
pH 6.0 PBS	100.0 ± 0.0	99.9 ± 0.2	100.0 ± 0.3	100.1 ± 0.2	99.6 ± 0.2	99.2 ± 0.3	98.9 ± 0.3	98.0 ± 0.3
pH 6.2 PBS	100.0 ± 0.0	100.0 ± 0.3	99.8 ± 0.2	99.8 ± 0.4	99.4 ± 0.2	99.0 ± 0.3	98.5 ± 0.3	96.3 ± 0.3
pH 6.4 PBS	100.0 ± 0.0	99.8 ± 0.1	99.7 ± 0.4	99.7 ± 0.3	99.2 ± 0.3	98.6 ± 0.2	97.1 ± 0.4	94.1 ± 0.5
pH 6.6 PBS	100.0 ± 0.0	99.8 ± 0.2	99.8 ± 0.5	99.4 ± 0.3	99.2 ± 0.4	97.7 ± 0.4	95.9 ± 0.7	93.5 ± 0.7
pH 6.8 PBS	100.0 ± 0.0	99.2 ± 0.4	98.5 ± 0.4	96.2 ± 0.4	93.3 ± 0.3	92.2 ± 0.5	88.5 ± 0.9	85.3 ± 1.1
pH 7.0 PBS	100.0 ± 0.0	96.5 ± 0.5	91.5 ± 0.6	89.1 ± 0.6	82.9 ± 1.0	78.0 ± 1.6	72.3 ± 2.4	65.7 ± 3.3

2.5. In vivo evaluation of formulations

2.5.1. In vivo elimination studies

In vivo pre-corneal drainage of each formulation was determined after instillation of 100 μL radiolabeled solution onto the left cornea using a gamma camera (Toshiba GCA 602A) adjusted to detect the radiation of ^{99m}Tc-DTPA and fitted with a 4-mm pinhole. The activity instilled ranged from 2.1 to 2.7 MBp per 100 μL dose. A small plastic vial containing a 100 μL aliquot of solution to be tested was placed near the eye of the rabbit, and used as a position reference. After instillation, the eyelids were kept closed for 5 s to prevent loss of the instilled solution. The rabbit was kept on a table without the rabbit restrainer, its head being supported by the experimenter's hand with its left eye in front of the collimator aperture at a distance of 5 cm.

Recording was started 5 s after instillation and frames were recorded over a period of 10 min using a 128 × 128 pixel matrix. Each formulation was tested on three rabbits. Individual 60 frames (a frame/5 s) were summed to obtain an overall picture of distribution of the label.

2.5.2. Ocular irritation studies

Ocular irritation studies were performed according to the Draize technique (Draize et al., 1944). A total of 6 rabbits, divided into 2 groups, were used. The Draize technique was used to assess acute, intermediate, and chronic exposure by applying compounds to the skin, penis, and eyes of rabbits. This technique has been used by the FDA to evaluate the safety of several substances (Draize et al., 1944; Fitzhugh et al., 1946). Baicalin in phosphate buffer at pH 5.8 (control solution) was instilled into the left eye, and formulation F1-3 into right eye, 0.1 mL every 3 h, five times a day for a period of 7 days. The condition of the ocular tissue was monitored after 1 h, 4 h 12 h, 24 h, 48 h and 72 h after the end of the last instillation. The conjunctival congestion, swelling and discharge were graded on scales from 0 to 3, 0 to 4 and 0 to 3, respectively. Iris hyperaemia and corneal opacity were graded on a scale from 0 to 4. The mean values from three treated eyes were calculated for each solution. The evaluation criteria used in accordance with the Draize technique were non-irritant from 0 to 3.9, slightly irritant from 4 to 8.9, moderately irritant from 9 to 12.9 and seriously irritant from 13 to 16.

2.5.3. Pharmacokinetic studies

Rabbits ($n = 3$) had been treated with 0.3% (w/v) ofloxacin ophthalmic solution for 4-day before surgery. Then the animals were anesthetized with lidocaine hydrochloride injection. A custom-designed LM-10 microdialysis probe (Bioanalytiacl systems, INC, USA) was implanted into the anterior chamber of each eye as described previously (Lonnroth et al., 1987). Probe inlet and outlet lines were tunneled beneath the conjunctiva, under the upper eyelid, and exited between the ears. The leads were protected with a latex glove pocket affixed to the top of the head. The probe was introduced as described previously (Duchêne and Wouessidjewe, 1996). The anchor was sutured to the sclera with 7-0 Vicryl, and conjunctiva was sutured over the anchor. Exterior wound surfaces were treated with 0.3% (w/v) ofloxacin ophthalmic solution. Animals were used for experimentation after 2 days recovery. Slit-lamp was taken after recovery to estimate fibrin formation and the condition of the eye prior to the use of rabbits in experiments.

Conscious rabbits ($n = 3$) were placed in rabbit restrainers which permitted free movement of the head. Following a 1-h equilibration period with perfusion of saline solution through the probe, different concentrations of standard baicalin saline solutions (4.00×10^{-2}, 8.00×10^{-2}, 1.60×10^{-1}, 3.20×10^{-1}, 6.40×10^{-1} and 1.00 μg mL^{-1}) were perfused through the probe at a rate of 3 μL min^{-1}, and dialysate were collected for 10 min after 30 min of perfusion. A 20 μL aliquot of each fraction was analyzed by HPLC (Shimadzu LC 10-AT, Japan).

After the disturbance of standard solutions was reduced to the negligible level by perfusion of saline solution through the probe 100 μL of Formulation F1-3 were dropped into the rabbit eyes. The samples were collected after every 10 min in the first hour and then after every 20 min until baicalin could not be detected. At the end of the experiment, euthanasia was performed under deep anesthesia with an intravenous injection of air through the marginal ear vein (De Lange et al., 2000).

3. Results and discussion

3.1. Selection of vehicle and dissolution medium

The pH of solutions plays a pivotal role in ophthalmic in situ pH-triggered gelling system. It contributes significantly to the stability of drugs and also influences the safety of products and the compliance of patients, hence in order to get the suitable pH for in situ gelling system, these three factors should be considered: (1) The tolerable pH of human eye: ophthalmic preparations requires strict to pH range, the tolerance pH for human eye is 5.0–9.0, and pH range of 6.0–8.0 is comfortable; (2) The acidity or basicity of excipients: Carbopol has acidic property, so it needs to be dissolved in a weak acidic solution to prepare a flowing liquid under non-physiological conditions; (3) The drug stability and solubility: baicalin is a weak acidic compound with several reaction points, which is not stable in strongly acidic or basic conditions. Baicalin would be hydrolyzed to form baicalin aglycone, and would be decomposed to polyhydroxy flavone aglycones by the baicalin enzymes contained in the root *S. baicalensis* (Qiu et al., 2004; Wang et al., 1990). Table 1 showed that the stability of baicalin was significantly influenced by different pHs of solutions. The baicalin solutions were stable in distilled water and phosphate buffer at pH 5.8, 6.0, 6.2, 6.4, 6.6, 6.8. And baicalin was unstable in pH 7.0 phosphate buffer obviously, which was just like in the pH 7.0 artificial tear fluid. The solubilities of baicalin in distilled water and in phosphate buffer at pH 5.8 and 6.8 were (0.057 ± 0.006) g mL^{-1}, (1.97 ± 0.017) g mL^{-1} and (10.70 ± 0.146) g mL^{-1}, respectively. Thus, baicalin would be dissolves in the phosphate buffer at pH 5.8 and 6.8.

Table 2
Combinations of Carbopol and HPMC E4M studied.

Formulation	Concentration (%, w/v)		Gelling capacity
	Carbopol 974P	HPMC E4M	
F1-1	0.3	0.2	–
F1-2	0.3	0.4	+
F1-3	0.3	0.6	++
F1-4	0.3	0.8	+++
F2-1	0.4	0.2	+
F2-2	0.4	0.4	++
F2-3	0.4	0.6	++
F2-4	0.4	0.8	+++
F3-1	0.5	0.2	+
F3-2	0.5	0.4	++
F3-3	0.5	0.6	+++
F3-4	0.5	0.8	+++
F4-1	0.6	0.2	+
F4-2	0.6	0.4	++
F4-3	0.6	0.6	+++
F4-4	0.6	0.8	+++

Note: –, no gelation; +, gels after a few minutes, dissolves rapidly; ++, gelation immediate, remains for few hours; +++, gelation immediate, remains for extended period.

According to the discussion and the results of the stability test and solubility test of baicalin, pH 5.8 was selected as the optimum pH value for in situ gelling system, and the phosphate buffer at pH 6.8 was considered as the receiving solution and the dissolution medium.

3.2. Formula optimization and drug release mechanism study

3.2.1. Gelling capacity study

Carbopol, the aqueous solution transforms into stiff gel when its pH is raised, was used as excipients in pH triggered in situ gelling system (Schoenwald et al., 1978). However, the concentration of Carbopol required for forming stiff gels results in highly acidic solutions which are not easily neutralized by the buffering action of the tear fluid. A reduction in Carbopol concentration without compromising the gelling capacity and rheological properties of the delivery systems may be achieved by the addition of viscosity enhancing polymers such as HPMC.

The formulation should have an optimum viscosity that will allow easy instillation into the eye as a liquid which would undergo a rapid sol-to-gel transition. Additionally, the gel formed in situ should preserve its integrity without dissolving or eroding for a prolonged period of time. In order to identify the compositions suitable for use as in situ gelling, gelling systems of various concentrations of Carbopol and HPMC were prepared and evaluated for gelling capacity. Table 2 shows that grade "++" of gelling capacity was more satisfactory.

3.2.2. In vitro release studies

The cumulative amount of baicalin released profiles for various formulations of in situ gelling system and baicalin in phosphate buffer at pH 5.8 (control solution) is shown in Fig. 1. All the solutions contained 0.1% (w/v) baicalin. For the control solution, almost all the baicalin released immediately after the start of release test. In the case of F2-2, more than 60% of the drug was released into the medium within 30 min and then gradually released to 90% in 6 h. For F2-3, F3-2 and F4-2, about 40% of baicalin was released into the medium within 30 min, and then to only 80% in 6 h. F1-3 had similar release trend as F2-3, F3-2 and F4-2 within 1 h, and the release rate became faster. There was 95% of baicalin release in 6 h. These results indicated that formulation F1-3 had relatively better sustained-release effect than the other formulations and can be used as ophthalmic sustained release drug delivery systems.

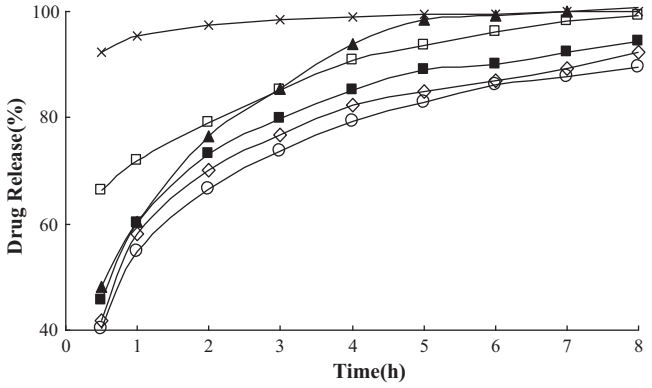

Fig. 1. Cumulative amount of baicalin released as a function of time from various formulations of in situ gelling system ($n = 3$). ▲:F1-3; □: F2-2; ■: F2-3; ◇: F3-2; ○: F4-2; ×: control solution.

3.2.3. Drug release mechanism

In order to investigate drug release mechanism, the release data were fitted to models representing first order, Higuchi and Ritger–Peppas equation. The linear regression analyses were summarized in Table 3. The examination of coefficient of determination (r^2) values for the formulation F1-3 indicated that Ritger–Peppas equation was a more suitable fit to drug release mechanism from in situ pH-triggered gelling system.

$$\text{Ritger-Peppas equation}: \quad F = KT^n \tag{1}$$

In Eq. (1), F is the fractional release of the drug; K is the proportionality constant; n is the diffusional exponent and T is the time. The diffusional exponent was calculated from the slope of the natural logarithmic values (ln) of the fractional release as a function of time (Table 3). The release mechanism for semisolid vehicles containing dissolved drug was found to be non-fickian or anomalous involving both diffusion and polymer relaxation ($0.5 < n < 1$). The data of release mechanism indicated that baicalin release was dependent on two simultaneous processes: water migration into the in situ gelling system and drug diffusion through continuously swelling gelling system. Similar result was also obtained by other researchers in other gelling system (Anumolu et al., 2009).

3.3. In vitro evaluation of formulations

3.3.1. Micro-morphology of in situ gelling system

As shown in Fig. 2(A), the bright green linear structures of in situ gelling system at pH 5.8 were observed. Fig. 2(B) shows that the color of FITC region was little faded and the linear structure was not changed after the gelling system was instilled with distilled water. And in Fig. 2(C), the micro-morphology of in situ gelling system clearly shows cluster structure instead of linear structure at pH 6.8. The images suggested that the structure of in situ gelling system was changed from pH 5.8 to pH 6.8. At the acidic condition, the Carbopol had linear structure and there were a lot of dissociation groups in the skeleton of Carbopol. But under physiological conditions, the molecular chains were extended by electrostatic repulsion of carboxyl groups, and the chance of becoming hydrogen bonds was increased producing a stronger gel.

Table 3
In vitro drug release kinetic of baicalin from floating in situ gelling system.

Formulation	First order	Higuchi	Ritger–Peppas	
	r^2	r^2	r^2	n
F1-3	0.8805	0.9582	0.9891	0.62

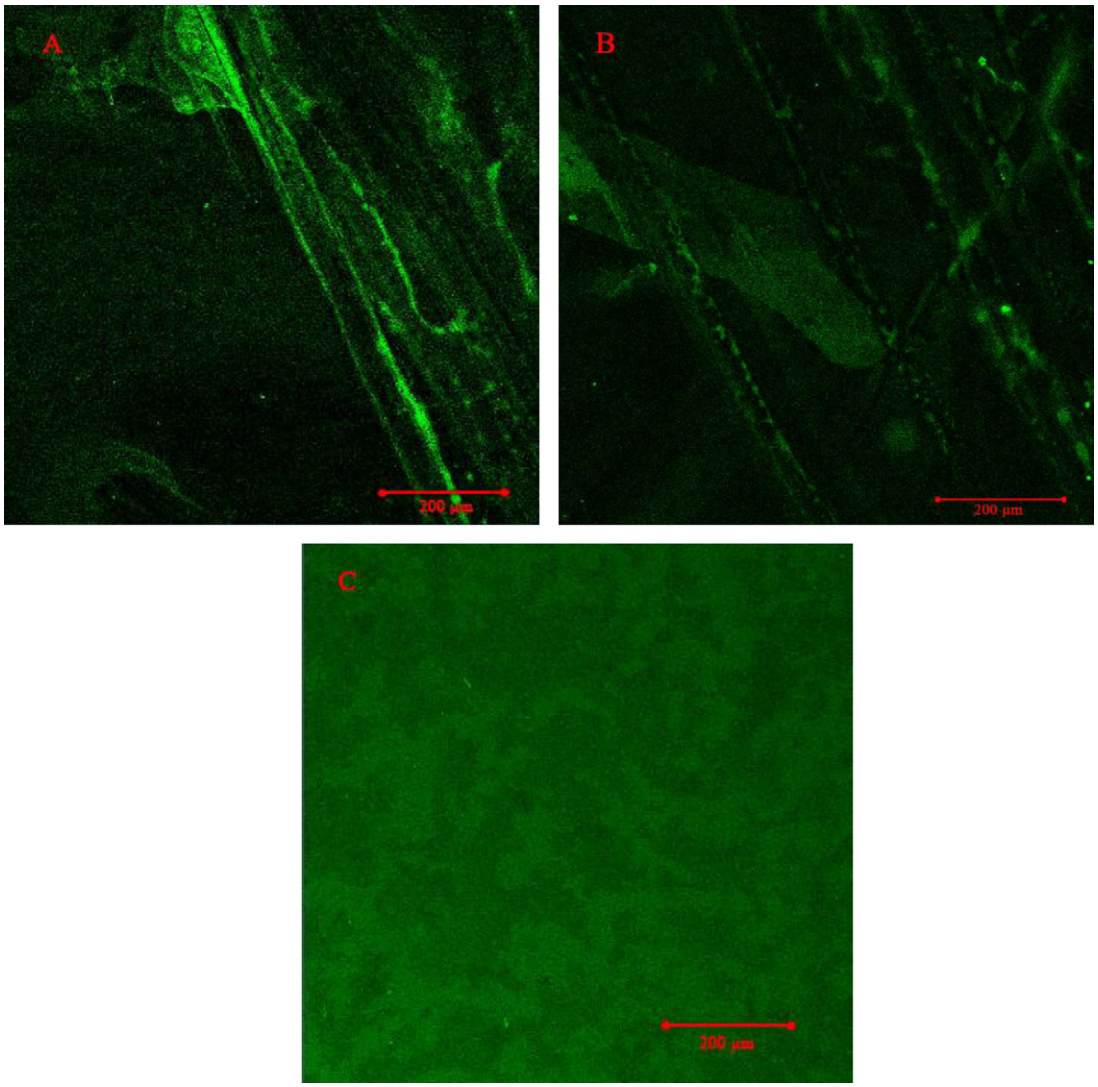

Fig. 2. CSLM micrograph of FITC-labeled in situ gelling system (formulation F1-3). (A) In situ gelling system under pH 5.8, (B) after instilled distilled water and (C) after instilled 0.2 M NaOH and adjusted pH to 6.8. Scale bar = 200 μm.

3.3.2. Rheological studies

The rheological behaviors of various gelling systems were investigated as a function of pH value. Fig. 3 illustrated the pH-induced viscosity change of formulation F1-3. The gelation pH value of polymer solution was pH 6.0. A sudden increase in the viscosity was observed after pH 6.0, suggesting the occurrence of sol–gel phase transition between these two conditions for in situ gelling system.

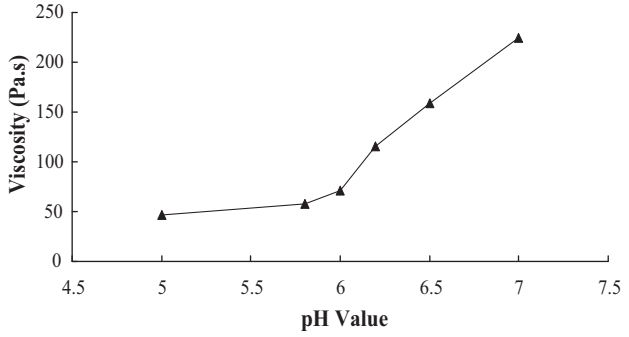

Fig. 3. pH-induced viscosity change of in situ gelling system (formulation F1-3).

3.3.2.1. Rheological behaviors of different polymer solutions without baicalin.

Fig. 4 shows the shear stress vs. shear rate flow curves of HPMC solution (0.6% (w/w)), Carbopol solution (0.3% (w/w)), and formulation F1-3 without baicalin (0.3% Carbopol-0.6% HPMC (w/v)) under non-physiological (25 °C, pH 5.8) and physiological (35 °C, pH 6.8) condition.

For HPMC solution, the shear stress increased linearly with increase in shear rate under non-physiological conditions, and the shear stress from physiological conditions to non-physiological conditions only had a slight decrease. The HPMC polymer solution under the two kinds of conditions did not undergo phase change to turn into a gel but remained as an easily flowing liquid, similar to pure water, which displays a Newtonian flow behavior (Miller and Drabik, 1984). On the other hand, the dilution by sodium hydroxide solution also had a great influence on the shear stress under physiological condition.

Fig. 4 also shows that, for Carbopol solution and formulation F1-3 without baicalin under non-physiological conditions as well as for HPMC solution under either non-physiological or physiological conditions, the shear stress increased linearly with an increase in shear rate, also demonstrating a Newtonian flow behavior (Miller and Drabik, 1984). However, under physiological conditions, the two solutions resisted the initial rotary motion, and the shear stress

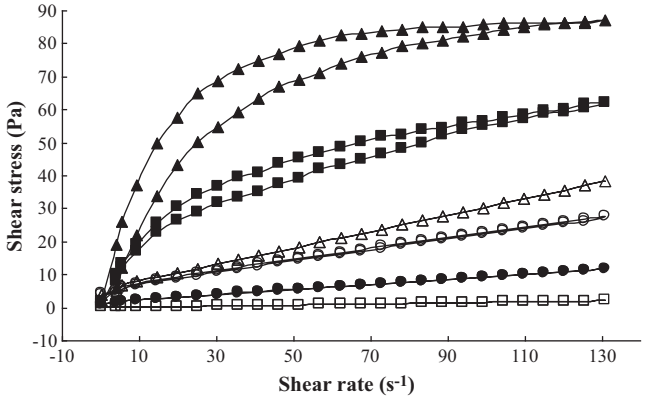

Fig. 4. Shear stress vs. shear rate flow curves of different polymer solutions without baicalin. ▲: formulation F1-3 without baicalin measure at 35 °C and pH 6.8; ■: 0.3% Carbopol solution measure at 35 °C and pH 6.8; □: formulation F1-3 without baicalin measure at 25 °C and pH 5.8; ◯: 0.6% HPMC solution measure at 25 °C and pH 5.8; ●: 0.6% HPMC solution measure at 35 °C and pH 6.8; □: 0.3% Carbopol solution measure at 25 °C and pH 5.8..

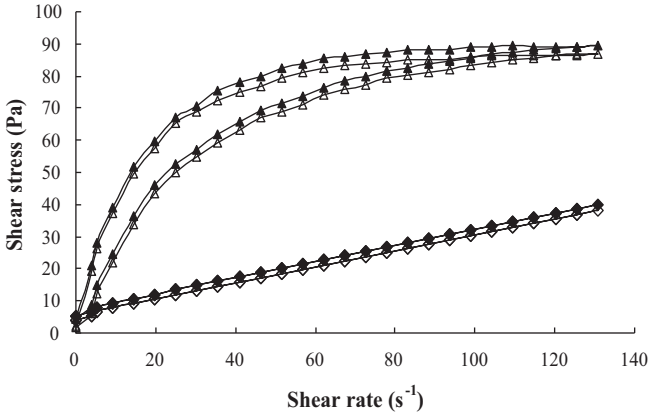

Fig. 5. Shear stress vs. shear rate flow curves of formulation F1-3 with and without baicalin. ▲: baicalin-containing F1-3 formulation measure at 35 °C and pH 6.8; △: formulation F1-3 without baicalin measure at 35 °C and pH 6.8; ♦: baicalin-containing F1-3 formulation at 25 °C and pH 5.8; ◇: formulation F1-3 without baicalin measure at 25 °C and pH 5.8.

increased suddenly at higher shear rate. The solutions began to flow after the shear stress reached its yield point. Therefore, the flow curve for these two solutions under physiological conditions exhibited pseudoplastic behavior with hysteresis (Lin and Sung, 2000; Patton and Robinson, 1975). At shear rate of 130 s^{-1}, the shear stresses of Carbopol and Formulation F1-3 without baicalin under physiological conditions were each approximately four to six times greater than those of under non-physiological conditions, suggesting significant sol–gel phase transition between these two conditions for both systems. The observed phase transitions for the two solutions were mediated by the variation of pH from 5.8 to 6.8 and can be attributed to the ionization of Carbopol polymer. At pH 6.8, the mutual repulsion of ionized carboxyl groups may produce more stretched Carbopol backbone and those carboxyl groups may also form stable hydrogen bonds with water molecules through hydrophilic interactions (Lochhead et al., 1989). On the other hand, the hydrophobic nature of Carbopol backbone may form hydrophobic interchain aggregation; this cross-linking phenomenon may result in the formation of more viscous gel at pH 6.8 environment (Wang et al., 1991).

Although the shear stress of Carbopol solution increased significantly under physiological conditions, a stronger gel can be formed by combining HPMC with Carbopol solutions. Fig. 4 shows that, under non-physiological and physiological conditions, the shear stresses of formulation F1-3 without baicalin were significantly greater than those of individual HPMC and Carbopol solution at each shear rate. This observation can be explained by the formation of crosslinks between the two polymers; that is, the water molecules may act as a crosslinking agent to form hydrogen bonds, which may lead to the formation of three-dimensional network and stronger gel (Kulicke and Nottelmann, 1989).

3.3.2.2. Rheological behaviors of formulation F1-3 with and without baicalin. In order to investigate the effects of baicalin on the rheological behaviors of the formulation, the rheological studies on formulation F1-3 with and without baicalin under non-physiological and physiological conditions were carried out. Fig. 5 demonstrated that formulation F1-3 with baicalin had similar flow behaviors as F1-3 formulation without baicalin, suggesting that the incorporation of baicalin did not disrupt the strong three-dimensional gel network formed under non-physiological and physiological conditions.

3.3.3. Accelerated stability studies

Accelerated stability studies were carried out on formulation F1-3. Table 4 shows that drug contents of formulation F1-3 were >98% and there were no change in pH (about 5.8), gelling capacity and in vitro release within 3 months.

3.4. In vivo evaluation of formulations

3.4.1. In vivo elimination studies

According to the activity of the radiation of ^{99m}Tc-DTPA over a period of 10 min, Fig. 6, which was the curves of the remaining radioactivity vs. the time, the in vivo elimination was discussed. The final images were divided into four regions of interest (ROIs), which were (0) the position reference, (1) the pre-corneal surface, (2) the lachrymal duct, and (3) the oropharynx. The curves illustrated that the radioactivity of the pre-corneal surface area of the control solution was delayed continuously, when compared with the radioactivity plateau phase of formulation F1-3. Furthermore, for the control solution, the radioactivity of the lachrymal duct and the oropharynx area increased at early period and decreased later; however, the radioactivity of formulation F1-3 was always higher than that of control solution.

The frames over the period of 10 min were divided into three phases, which were prophase, metaphase and anaphase. Fig. 7 shows the comparison of the scintigraphic images between the control solution and formulation F1-3 during three phases.

As shown in Fig. 7, the control solution flowed through the lachrymal duct, and the radiolabeled solution had already reached the oropharynx at metaphase, and diffused into capillaries at anaphase. Meanwhile, formulation F1-3 flowed slower than the control solution, and the diffusion was not obviously watched at metaphase and anaphase.

It was reported that Carbopol resins, an acrylic-acid based polymers, are available in a range of molecular weights and may be linear, branched or cross-linked (Robinson and Mlynek, 1995), have

Table 4
The drug content (%) of stability studies for 3 months (mean ± SD, $n = 3$).

Formulation F1-3	Months			
	0	1	2	3
4 ± 1 °C	100.0 ± 0.0	100.0 ± 0.3	99.8 ± 0.5	99.4 ± 0.5
25 ± 1 °C	100.0 ± 0.0	100.3 ± 0.4	99.4 ± 0.7	98.8 ± 1.3
45 ± 1 °C	100.0 ± 0.0	99.3 ± 0.7	98.9 ± 0.5	98.2 ± 0.8

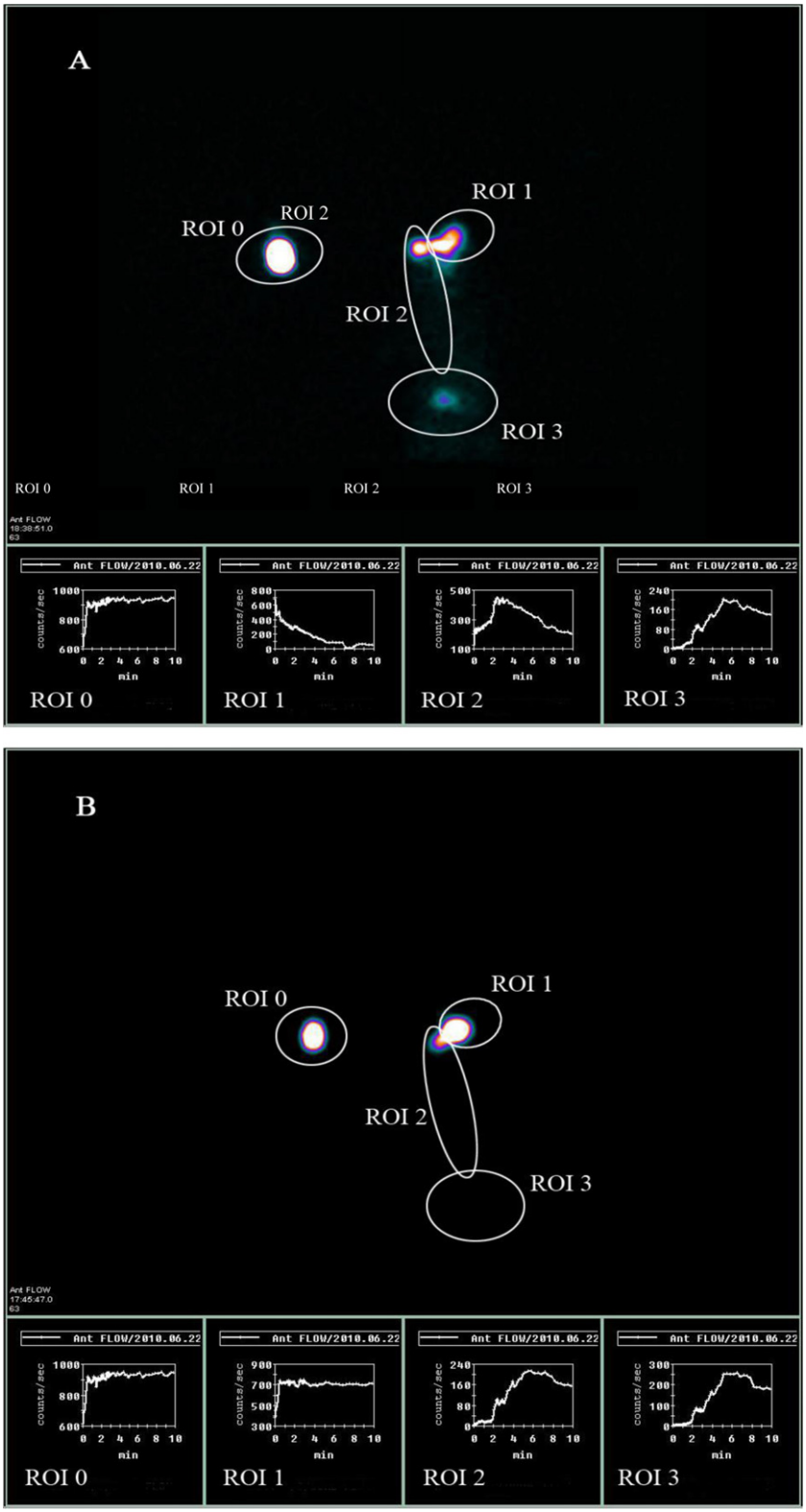

Fig. 6. The curves of the remaining activity vs. the time (A): in vivo elimination of the control solution and (B): formulation F1-3. Typical scintigraphic images divided into four regions of interest (ROIs): (0) the position reference, (1) the pre-corneal surface, (2) the lachrymal duct, and (3) the oropharynx..

been investigated very frequently for the development of ocular drug delivery systems owing to their excellent mucoadhesive property. So according to the results of in vivo elimination studies, formulation F1-3 had better ability to retain drugs than the control solution.

3.4.2. Ocular irritation studies

The average score of the control solution and formulation F1-3 were 0, which indicated that all formulations were non-irritant. Excellent ocular tolerance was noted. No visible ocular damage

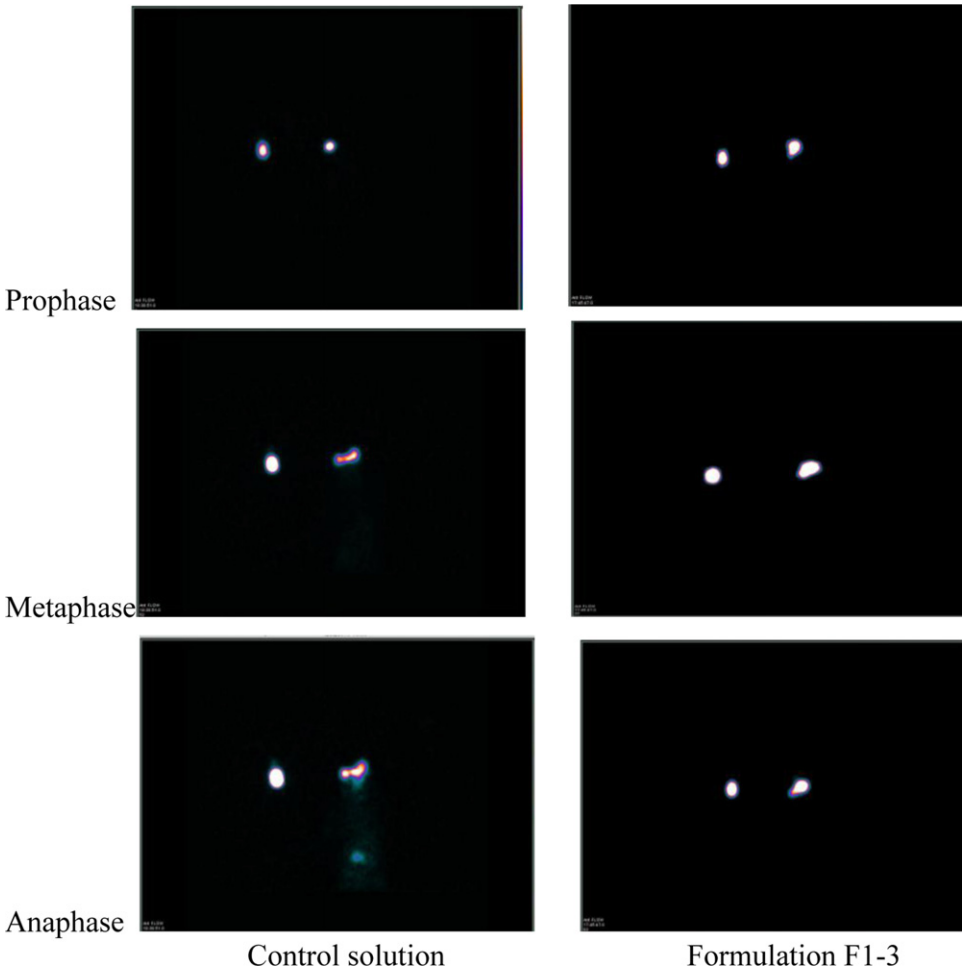

Fig. 7. The comparison of the scintigraphic images between the control solution and formulation F1-3.

or abnormal clinical signs to the cornea, iris or conjunctivae were observed.

3.4.3. Pharmacokinetic studies

In vivo recovery was defined as (Higuchi, 1960):

$$R = \frac{C_{in} - C_{out}}{C_m - C_{out}} \quad (2)$$

where C_{in} is the concentration of standard solutions, C_{out} is the concentration of dialysate and C_m is the concentration in aqueous humor. A linear equation was plotted by $(C_{in} - C_{out})$ vs. C_{out}, and the slope of the line gave the recovery (R).

As it was showed in Fig. 8, the linear regression between perfusate (C_{in}) and dialysate (C_{out}): $C_{in} - C_{out} = -0.4656\,C_{out} + 3.2127$ ($R^2 = 0.9994$), so the recovery (R) in vivo was $(44.41 \pm 6.49)\%$. Perfusion flow-rate, temperature, perfusate composition, characteristics of the drug, characteristics of the semipermeable membrane, and the surface of the semipermeable membrane may all affect recovery. All parameters that influence in vitro recovery will also influence in vivo recovery. However, tissue characteristics will play an important role and may ultimately determine the recovery. In vivo recovery depends on the diffusion in three regions: probe lumen, dialysis membrane and periprobe environment (Benveniste et al., 1991; Bungay et al., 1990). Diffusion in probe lumen is limited only with the use of very low flow rates. Diffusion through the dialysis membrane is limited only when transport through the periprobe environment is rapid. Rapid diffusion through the periprobe environment occurs in most flowing systems. In tissues, effective diffusion through the extracellular fluid determines the recovery of the microdialysis probes (Morrison et al., 1991).

Aqueous humor pharmacokinetic parameters were presented in Table 5 and Fig. 9. It can be seen that the AUC value of formulation F1-3 was much higher than that of the control solution, which were 6.1-fold vs. the control group ($p < 0.01$), and the C_{max} value of formulation F1-3 vs. the control solution was 3.6-fold ($p < 0.05$). The T_{max} value and $t_{1/2}$ value of formulation F1-3 were higher than those of control solution, which were 2.6-fold. These in vivo results

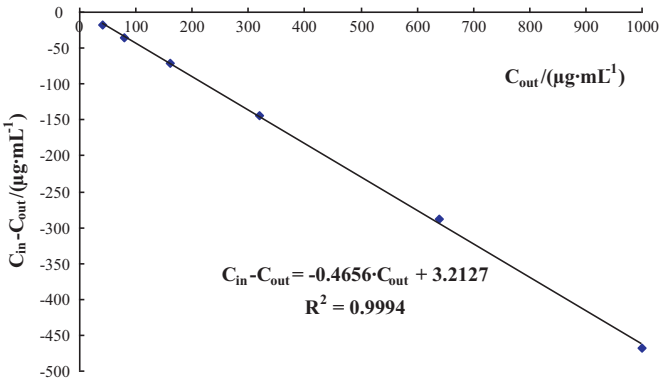

Fig. 8. In vivo recovery of microdialysis probe in aqueous humor ($n = 3$).

Table 5
Pharmacokinetics parameters of baicalin in aqueous humor after topical administration rabbit (mean ± SD, $n = 3$).

Formulation	AUC/($\mu g\,mL^{-1}\,h$)	C_{max}/($\mu g\,mL^{-1}$)	T_{max}/h	$t_{1/2}$/h
Control	0.0360 ± 0.0055	0.0922 ± 0.0282	0.167 ± 0	0.4845 ± 0.2422
F1-3	0.2210 ± 0.0574**	0.3330 ± 0.1159*	0.444 ± 0.096**	1.2608 ± 0.4310*

Sign: control, baicalin phosphate buffer at pH 5.8.
* $p < 0.05$ vs. control.
** $p < 0.01$ vs. control.

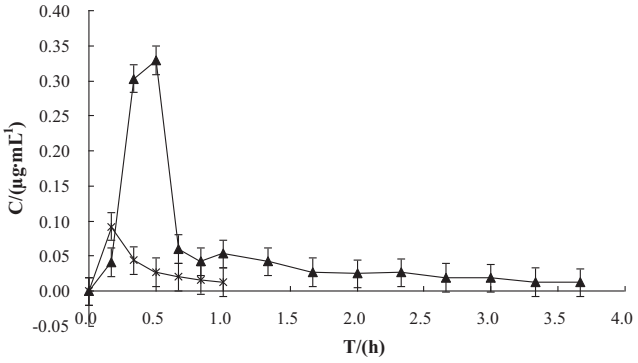

Fig. 9. Aqueous humor baicalin concentration–time profiles following a 100-μL topical dose in conscious rabbits ($n = 3$). ▲: Formulation F1-3; ×: The control solution.

demonstrated further that in situ gelling system, for its ability to sustain drug release, can be used as the ideal ocular delivery system with improved the ocular bioavailability.

4. Conclusion

Baicalin was successfully formulated in pH-triggered in situ gelling system using Carbopol® 974P (0.3%, w/v) as a pH-triggered gelling agent in combination with HPMC E4M (0.6%, w/v) as a viscosity enhancing agent. It was found that the gelling system can flow easily under non-physiological condition (25 °C, pH 5.8) and undergo rapid gelation under physiological condition (35 °C, pH 6.8). The in situ gelling system can support sustained drug release over an 8-h period, and the release mechanism in vitro was dependent on two simultaneous processes: water migration into the in situ gelling system and drug diffusion. Stability data recorded over a 3-month period under (4 ± 1) °C, room temperature (25 ± 1) °C, and accelerated temperature (45 ± 1) °C condition indicated that the formulation was stable. And the formulation caused no irritation to rabbit eye tissues. Both the in vitro and the in vivo results indicated that the in situ pH-triggered gelling system is a viable alternative to conventional eye drops by virtue of its ability to enhance bioavailability through its longer precorneal residence time and the ability to sustain drug release. More importantly, it was a suitable medium for baicalin, the pH-sensitive drug, to be used as novel ophthalmic delivery system.

Acknowledgments

This study was supported by International Science Cooperation Project of China (No.2007DFC31670), National Key Technology Research and Development Program of China (2009ZX09311-002), Chinapostdoctor Special Funded Project (2010003304) and Supported by Program for Changjiang Scholars and Innovative Research Team in University.

References

Anumolu, S., Singh, Y., Gao, D., 2009. Design and evaluation of novel fast forming pilocarpine-loaded ocular hydrogels for sustained pharmacological response. J. Control Rel. 137, 152–159.
Baudouin, C., 1996. Side effects of antiglaucomatous drugs on the ocular surface. Curr. Opin. Ophthalmol. 7, 80–86.
Benveniste, H., Hansen, A.J., Ottosen, N.S., 1991. Determination of brain interstitial concentrations by microdialysis. J. Neurochem. 57, 1741–1750.
Bungay, P.M., Morrison, P.F., Dedrick, R.L., 1990. Steady-state theory for quantitative microdialysis of solutes and water in vivo and in vitro. Life Sci. 46, 105–119.
Cheng, G.Q., Feng, N.P., Tang, Q.W., Li, L., 2001. Studies on in vitro antibacterial action of baicalin in vitro. J. Chin. Hosp. Pharm. J. 21, 347–348.
De Lange, E.C., De Boer, A.G., Breimer, D.D., 2000. Methodological issues in microdialysis sampling for pharmacokinetic studies. J. Adv. Drug Deliv. Res. 45, 125–148.
Draize, J.H., Woodard, G., Calvery, H.O., 1944. Methods for the study of irritation and toxicity of substances. J. Pharmacol. Exp. Ther. 82, 377–390.
Duchêne, D., Wouessidjewe, D., 1996. Pharmaceutical and medical applications of cyclodextrins. In: Dumitriu, S. (Ed.), Polysaccharides in Medical Applications. Marcel Dekker, New York, pp. 575–602.
Fitzhugh, O.G., Woodard, G., Braun, H.A., 1946. The toxicities of compounds related to 2, 3-dimercaptopropanol (BAL) with a note on their relative therapeutic efficiency. J. Pharm. Exp. Ther. 87, 23–27.
Jung, S.H., Kang, K.D., Ji, D., Fawcett, R.J., 2008. The flavonoid baicalin counteracts ischemic and oxidative insults to retinal cells and lipid peroxidation to brain membranes. Neurochem. Int. 53, 325–337.
Higuchi, T., 1960. Physical chemical analysis of percutaneous absorption process from creams and ointments. J. Soc. Cosmet. Chem. 11, 85–97.
Huang, H., Yang, A.X., Li, D., 2009. Baicalin suppresses expression of Chlamydia protease-like activity factor in Hep-2 cells infected by *Chlamydia trachomatis*. Fitoterapia 80, 448–452.
Kulicke, W.M., Nottelmann, H., 1989. Polymers in aqueous media: performance through association. In: Glass, J.E. (Ed.), Advances in Chemistry Series, vol. 223. American Chemical Society, Washington, DC, pp. 15–44.
Lin, H.R., Sung, K.C., 2000. Carbopol/pluronic phase change solutions for ophthalmic drug delivery. J. Control Rel. 69, 379–388.
Lochhead, R.Y., Davidson, J.A., Thomas, G.M., 1989. Polymers in aqueous media: performance through association. In: Glass, J.E. (Ed.), Advances in Chemistry Series, vol. 223. American Chemical Society, Washington DC, pp. 113–147.
Lonnroth, P., Jansson, P.A., Smith, U., 1987. A microdialysis method allowing characterization of intercellular water space in humans. Am. J. Physiol. 253, 228–231.
Maurice, D.M., 1987. In: Saettone, M.F., Bucci, M., Speiser, P. (Eds.), Ophthalmic Drug Delivery: Biopharmaceutical, Technological and Clinical Aspects, vol. 11. Liviana Press, Padova, pp. 19–26.
Miller, S.C., Drabik, B.R., 1984. Rheological properties of poloxamer vehicles. Int. J. Pharm. 18, 269–276.
Morrison, P.F., Bungay, P.M., Hsiao, J.K., Ball, B.A., Mefford, I.N., Dedrick, R.L., 1991. Quantitative microdialysis: analysis of transient and application to pharmacokinetics in brain. J. Neurochem. 57, 103–119.
Nakamura, N., Hayasaka, S.J., Zhang, X.Y., 2003. Effects of baicalin, baicalein, and wogonin on interleukin-6 and interleukin-8 expression, and nuclear factor-kb binding activities induced by interleukin-1b in human retinal pigment epithelial cell line. Exp. Eye Res. 77, 195–202.
Nanjawade, B.K., Manvi, F.V., Manjappa, A.S., 2007. In situ-forming hydrogels for sustained ophthalmic drug delivery. J. Control Rel. 122, 119–134.
Patton, T.F., Robinson, J.R., 1975. Ocular evaluation of polyvinyl alcohol vehicle in rabbits. J. Pharm. Sci. 64, 1312–1316.
Qi, L., Zhou, R., Wang, Y.F., Zhu, Y.C., 1998. Study of major flavonoids in crude Scutellariae radix by micellar electrokinetic capillary chromatography. J Capillary Electrophor. 5, 181–183.
Qiu, F., Tang, X., He, Z.G., Li, H.Z., 2004. Stability of baicalin aqueous solution by validated RP-HPLC. J. Chin. Pharm. Sci. 13, 134–137.
Robinson, J.R., Mlynek, G.M., 1995. Bioadhesive and phase-change polymers for ocular drug delivery. Adv. Drug Deliv. Rev. 16, 45–50.
Salminen, L., 1990. Review: systemic absorption of topically applied ocular drugs in humans. J. Ocul. Pharmacol. 6, 243–249.
Schoenwald, R.D., Ward, R.L., DeSantis, L.M., Roehrs, R.E., 1978. Influence of high-viscosity vehicles on miotic effect of pilocarpine. J. Pharm. Sci. 67, 1280–1283.
Sieg, J.W., Robinson, J.R., 1977. Vehicle effects on ocular drug bioavailability II: evaluation of pilocarpine. J. Pharm. Sci. 66, 1222–1228.
Srividya, B., Cardoza, R.M., Amin, P.D., 2001. Sustained ophthalmic delivery of ofloxacin from a pH triggered in situ gelling system. J. Control Rel..

Topalkara, A., Güler, C., Arici, D.S., Arici, M.K., 2000. Adverse effects of topical antiglaucoma drugs on the ocular surface. Clin. Exp. Ophthalmol. 28, 113–117.

Tromp, R.H., van de Velde, F., van Riel, J., Paques, M., 2001. Confocal scanning light microscopy (CSLM) on mixtures of gelatine and polysaccharides. J. Food Res. Int. 34, 931–9381.

Wang, T.K., Iliopoulos, I., Audebert, R., 1991. Water-soluble polymers: synthesis, solution properties, and applications. In: Shalaby, S.W., McCormick, C.L., Butler, G.B. (Eds.), ACS Symposium Series 467. American Chemical Society, Calorimetry, J. Phys., Washington, DC, pp. 218–231.

Wang, X., Luo, X., Bi, K.S., Hong, F.S., Jin, Z.Y., Dong, H.R., 1990. A modified method for assaying scutellarin in Radixscutellariaein Chinese Pharmacopoeia. J. Shenyang Coll. Pharm. 7, 208–211.