—Regular Articles—

Preparation and Evaluation of a Carbopol®/HPMC-based *In Situ* Gelling Ophthalmic System for Puerarin

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The purpose of this study was to develop a pH-triggered *in situ* gelling vehicle for ophthalmic delivery of puerarin. The effect of hydroxypropyl-β-cyclodextrin (HP-β-CD) on the aqueous solubility and *in vitro* corneal permeation of puerarin was also investigated. The puerarin solubility increased linearly and proportionally to the HP-β-CD concentrations and 5% (w/v) HP-β-CD enhanced its ocular permeability significantly. Carbopol® 980NF was used as the gelling agent in combination with HPMC (Methocel E4M) which acted as a viscosity-enhancing agent. The optimum concentrations of Carbopol® 980NF and HPMC E4M for the *in situ* gel-forming delivery systems were 0.1% (w/v) and 0.4% (w/v), respectively. When these two vehicles were combined, an *in situ* gel that had the appropriate gel strength and gelling capacity under physiological condition could be obtained. This combined solution could flow freely under non-physiological condition and showed the character of pseudoplastic fluid under both conditions. Both *in vitro* release studies and *in vivo* pharmacokinetics studies indicated that the combined polymer systems performed better in retaining puerarin than puerarin eye drops did. These results demonstrate that the Carbopol® 980NF/HPMC E4M can be a viable alternative to conventional puerarin eye drops to enhance ocular bioavailability and patient compliance.

Key words——ophthalmic delivery system; *in situ* gelling; Carbopol®; HPMC; puerarin; hydroxypropyl-β-cyclodextrin (HP-β-CD)

INTRODUCTION

Puerarin is an osajin isoflavone extracted from the radix of *Pueraria lobata* (Willd.) Ohwi.[1] It is a white needle-like crystal and poor in water solubility. Reportedly, puerarin blocks β acceptors, lowers intraocular pressure, improves ocular blood flow and ameliorates retinal function and microcirculation.[2—4] Therefore, puerarin is used as a therapeutic agent for cataracta glauca, ocular hypertension, etc. In China, puerarin eye drops are available commercially, and its standard is recorded in the National Drug Standard of China Food and Drug Administration as WS_1-(X-111)-2003Z. According to Wu and coworkers, puerarin drops depress intraocular pressure in a manner similar to that of the Timolol ophthamalic solution. Although puerarin is weaker than Timolol in lowering the intraocular tension quickly, it can maintain intraocular hypotension for a longer time period.[5]

Due to the lachrymation, the normal tear turnover and the drainage from the nasolacrymal duct, the puerarin eye drops eliminate rapidly, which causes a short precorneal residence time and a limitation of transcorneal absorption. Moreover, if the puerarin is eliminated via nasolacrymal duct to the gastrointestinal tract, it may cause some respiratory and gastrointestinal side effects. Therefore, prolonged exposure of the cornea to the applied drug is vital.

A significant increase in the precorneal residence time of drugs and consequent bioavailability can be achieved by using delivery systems based on the concept of *in situ* gel formation. These systems consist of polymers that exhibit sol-to-gel phase transitions due to a change in a specific physico-chemical parameter (for example, pH or temperature). Depending on the method employed to cause a sol-to-gel phase transition on the eye surface, the following three types of systems are recognized: pH triggered, temperature-dependent and ion-activated.[6—9] Carbopol® is a polyacrylic acid (PAA) polymer, which shows a sol to gel transition in aqueous solution as the pH is raised above its pK of about 5.5, and it is also generally considered to be very mucoadhesive.[9,10] However, as the concentration of Carbopol® increases in the vehicle, its acidic nature may cause stimulation to the eye tis-

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sue. To reduce the Carbopol® concentration without compromising the *in situ* gelling properties as well as overall rheological behaviors, a suitable viscosity-enhancing polymer is usually added into the formulation.[7,9,11]

Another problem with puerarin administered as aqueous eye drops is obtaining the desired drug concentration. To achieve a satisfactory curative effect, 1 % (w/v) puerarin is used in ocular preparations as an effective drug delivery system. However, due to the poor water solubility, a solubilizing agent is required.

Hydroxypropyl-β-cyclodextrin (HP-β-CD) is a hydroxypropyl derivant of β-cyclodextrin. It can increase aqueous solubility of some lipophilic water insoluble drugs without changing their molecular structure and on the other hand, it can also act as penetration enhancer by increasing drug availability at the surface of the biological barrier. HP-β-CD is the first β-cyclodextrin derivant supplied via intravenous injection that is approved by U.S. Food and Drug Administration (FDA). In particular, HP-β-CD is most commonly applied in aqueous eye drop formulations because of lower toxicity compared with parent CDs.[12,13]

In this paper, the effect of HP-β-CD on the aqueous solubility and *in vitro* corneal permeation of puerarin was investigated to achieve the curative concentration and enhance its ocular bioavailability. A pH-triggered *in situ* gel for puerarin was developed and Carbopol® was used as the gelling agent in combination with hydroxypropylmethylcellulose (HPMC) which acted as a viscosity-enhancing agent. The rheological behaviors of various aqueous polymer solutions under non-physiological and physiological conditions were evaluated. In addition, the *in vitro* puerarin release and *in vivo* puerarin pharmacokinetics of the drug-containing polymer solutions and eye drops were characterized to evaluate sustained release effect of the *in situ* gelling polymer solutions.

EXPERIMENTAL

Materials and Reagents Puerarin was supplied by Sichuan Yuxin Pharmaceutical Co., Ltd. (Sichuan, China). HP-β-CD was purchased from Xi'an Deli Biology & Chemical Industry Co., Ltd. (Shanxi, China). Carbopol® (980 NF, Goodrich) and HPMC (E4M, Methocel) were provided as gifts. The polyvinylpyrrolidone (PVP) K30 was purchased from Jinyu Fine Chemicals Co., Ltd. (Tianjin, China). Chemical Reference Standards (CRS): puerarin (110752—200209, for assaying) was supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol was chromatographic grade (Fisher Chemicals) and water was bi-distilled. All the other reagents are of analytical grade.

Animals Male and female New Zealand albino rabbits were obtained from the nursery of the Experimental Animal Professional Committee, Sichuan, China (License No.: SCXK (111) 2004-14). The experimental animals weighed between 2.5 and 3.0 kg, were individually housed in an air-conditioned and light-controlled room at 25 ± 1°C and at 70 ± 5% relative humidity. They were given a standard pellet diet and were provided with water *ad libitum*. All animals were healthy and free of clinically observable ocular abnormalities. The local ethics committees for animal experimentation approved all experiments.

Phase-solubility Studies Solubility studies were conducted according to Higuchi and Lach.[14] To determine the solubility of puerarin, excess drug (300 mg) was added to 5 ml of bi-distilled water containing different concentrations of HP-β-CD (from 0 to 20% (w/v)). The suspensions were vortexed for 10 min in 10 ml screw-capped test-tubes, and then were stirred in a water bath (25 ± 1°C) for 72 h to establish equilibrium. Each sample was filtered through a 0.45 μm micropore film and the concentration of puerarin in the filtrate was determined by high performance liquid chromatography (HPLC).

***In Vitro* Permeability Studies** The experiments were performed according to the methods reported by others.[15] Vilia-Chien perfusion chambers were used, which include donor and reservoir chambers. The glutathione bicarbonate Ringer (GBR) buffer was used as a releasing medium.[16] The rabbits were euthanized with an intravenous lethal dose of sodium pentobarbital. The whole eyes were enucleated from their sockets and the corneas with 2 mm ring of sclera were immediately excised, where the corneal area available for diffusion was 0.795 cm^2. Various ocular tissues were dissected from the corneas, which then were washed with distilled water, and preserved in the GBR buffer. The penetrative experiments *in vitro* must be initiated within 20 min of dissection. The isolated corneas were placed and clamped between two compartments of the perfusion chambers, so that the epidermis faced the donor chamber. Four milliliter

preheated (34℃) GBR buffer was added to the reservoir chamber and an equal volume of drug solution in GBR buffer was then added to the donor chamber. The perfusion chambers were placed on the magnetic stirrer and thermostated by circulating at a constant water temperature 34±0.5℃ (the surface temperature of the cornea). To insure mixing and oxygenation, an O_2 : CO_2 (95 : 5) mixture was bubbled through each compartment at a rate of 2—3 bubble/s aided by magnetic stir bars. We removed 200 μl of the solution from the reservoir chamber at 15, 30, 45, 60, 90, 120, 150, 180, 240 and 300 min with the precision micro liter pipette. Each sample was immediately replaced with an equal volume of GBR buffer to maintain a constant volume. The sample solution was centrifuged (10000 r/min, 10 min). The amount of puerarin permeated across the cornea was assayed with HPLC and permeability coefficient was calculated.

The corneal hydration level evaluation was performed according to the methods reported by Saettone and colleagues.[17] Wet corneal weight (W_w) was obtained after careful removed of the scleral tissue; each cornea was then desiccated at 70℃ overnight to give the corresponding dry corneal weight (W_d). The percent corneal hydration level (%HL) was defined as $(W_w\text{-}W_d)/W_w \times 100$. These were performed on untreated corneas (removed no later than 20 min after the animals' death) and on corneas recovered from permeation tests.

Sample Preparation The preparation instruction for the pH-triggered *in situ* gel-forming system is shown as outlined below: The HP-β-CD was dissolved in 75 ml bi-distilled water and then puerarin was added and stirred continuously until dissolved. Mannitol and ethylparaben were added later, which were used as isotonicity agent and preservative, respectively. After dissolved, HPMC was added and allowed to hydrate and swell. The Carbopol® was sprinkled over this solution and allowed to hydrate overnight. The desirable pH was adjusted by 0.5 mol/l NaOH and then the solution was stirred constantly until a uniform solution was obtained. Finally, bi-distilled water was added to make the volume to 100 ml.

In order to identify the pH of the polymer solution and the concentrations of Carbopol® and HPMC suitable for use as *in situ* gelling systems, the dynamic viscosity of the gel solution was measured under non-physiological condition (pH 4.3, 4.8 or 5.3 and 25℃) and physiological condition (pH 7.4 and 34℃) using a rotating cylinder viscometer (Shanghai Precision Instrumentation Co., Ltd., China). The gelling capacity of the polymer solution containing different ratio of Carbopol® and HPMC was also evaluated and it was performed by placing a drop of the polymer solution in a vial containing 2 ml of artificial tear fluid (STF) freshly prepared and equilibrated at 34℃ and visually assessing the gel formation and noting the time for gelation and the time taken for the gel formed to dissolve. The composition of STF was sodium chloride 0.670 g, sodium bicarbonate 0.200 g, calcium chloride·$2H_2O$ 0.008 g, and bi-distilled water q.s. 100.0 g.[18] Physiological pH (7.4 ±0.1) was adjusted by adding the required amount of 0.1 N HCl.

Rheological Studies The rheology of gel solution under the different shear rates (6, 12, 30 and 60 r/min) was measured under non-physiological condition (pH 4.8 and 25℃) and physiological condition (pH 7.4 and 34℃), respectively.

***In Vitro* Release Studies** According to several reports,[7,9] the *in vitro* release of puerarin from the formulations was studied through a cellophane membrane fixed in a self-made polytef sample cell. The *in vitro* drug release from the various polymer solutions was first conducted by filling 1 g of puerarin-containing polymer solution into small, circular polytef containers (4.0 cm i.d. and 0.9 cm in depth) in triplicate and placing each container in a 1000 ml beaker. Care was taken to make sure that no air bubbles were inside the solutions. The beaker was then filled with 500 ml STF. The experiments were performed in a dissolution testing apparatus (ZRS-8G, Tianjin University Precision Instrument Factory, Tianjin, China). The beaker was placed in a circulating water bath with stirring rods to stir the release medium. The temperature and stirring rate were maintained at 34℃ and 50 r/min, respectively. Aliquots (5 ml) were withdrawn from the release mediums at each sampling time and replaced by an equal volume of the release medium. The samples were filtered through 0.45 μm syringe filters, and were subjected to HPLC analysis to determine the puerarin concentrations.

***In Vivo* Evaluation** 50 μl of the test solutions was instilled into the conjunctival sac of albino rabbits. For each formulation four animals were then killed at each of the following time intervals: 0.5, 1, 2, 3, 4, 6, 8, 10, 16, and 24 h post instillation. Aqueous

humor (200 μl) was withdrawn with a 27-gauge, 1.3 cm needle attached to 1 ml disposable syringe inserted through the corneal-scleral junction and slightly upwards into the anterior chamber. The collected aqueous humor was centrifuged at 10000 r/min for 10 min. Twenty five microliter of supernatant was injected into the HPLC system.

HPLC Conditions The chromatographic system consisted of LC-2010A automatic HPLC (Shimadzu, Japan) and a SPD-M10A*vp* diode array detector (Shimadzu, Japan). The HPLC separation was performed on reversed phase C18 columns (5 μm, 150×4.6 mm, Kromasil, Sweden). The mobile phase consisted of a mixture of methanol and 0.1% citric acid (25 : 75, v/v). The mobile phase was filtered through a 0.45 μm microporous membrane and was deaerated ultrasonically prior to use. The detector was set at 250 nm and the flow rate was set at 1 ml/min. The column temperature was set at 30℃.

RESULTS AND DISCUSSION

HPLC Method Validation We validated the HPLC method by linearity, precision (repeatability and intermediate precision), accuracy and limit of detection. The puerarin standard curve was linear over the concentration range of 0.5—20 μg/ml (r= 0.9999). The repeatability was evaluated by five replicate determinations of solutions within a day with three different puerarin concentrations. The relative standard deviation (RSD) obtained for 0.5 μg/ml, 5 μg/ml and 20 μg/ml puerarin solutions were 1.64%, 1.05% and 2.18%, respectively. The intermediate precision determined by analyzing these solutions over five consecutive days was reproducible, with RSDs of 2.04%, 1.43% and 1.11%. The accuracy, expressed as recovery percentage of puerarin from blank gel matrix, was assessed by spiking blank gel matrix with puerarin in 10 mg/ml concentration and found to be 99.5±2.67% (n=5). The detection limit for the assay was estimated to be 0.01 mg/ml.

Phase-Solubility Studies The phase-solubility diagram of puerarin with HP-β-CD at 25±1℃ is plotted according to Higuchi and his coworkers (Fig. 1).[14, 19] In this figure, the puerarin solubility coefficient increases linearly and proportionally to the HP-β-CD concentrations. Therefore, the solution curve is classifiable as A_L type. The apparent stability constant of puerarin (k) was calculated from the straight line of the diagram according to the following

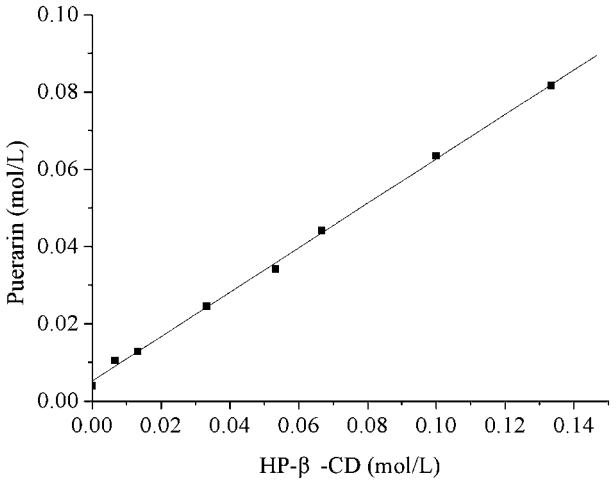

Fig. 1. Phase-solubility Graph of Puerarin in HP-β-CD

equation:[20]

$$k=S/[Cs(1-S)]$$

Wherein, Cs (the intercept) denotes the solubility of puerarin in the absence of HP-β-CD and S denotes the slope of the straight line. The value of k was found to be 266.26 1/M. The k value between 200 and 5000 1/M is considered by various authors as the most suitable for improving of the bioavailability of poorly soluble drugs.[20]

As puerarin is not highly water-soluble, PVP is generally used as solubilizer for 1% puerarin eye drops. The dissolving effect of PVP and other macromolecules on puerarin was studied by Wu.[21] The result showed that 4.3% PVP was the most suitable for improving puerarin solubility (1.30%). This experiment indicates that the solubility of puerarin can be increased to 1.02% with 5% (w/v) HP-β-CD, 1.84 % with 10% (w/v) HP-β-CD, and the solubility of puerarin increased linearly as the HP-β-CD concentration increased. Thus, it demonstrates the preparation of 1% or higher puerarin concentration in eye drop formulations can be realized with HP-β-CD as solubilizer.

***In Vitro* Permeability Studies** The accumulated release amounts of puerarin passing through the cornea were calculated by the following formula:

$$Q=V_0\left(C_n+\frac{V}{V_0}\sum_{i=1}^{n-1}C_i\right)=V_0C_n+V\sum_{i=1}^{n-1}C_i$$

(Q: the accumulated release amount within time t, C_n: the measured concentration value within time t, V_0: the total volume of solution in the reservoir chamber, V: the sampling volume per time point, C_i: the measured value of concentration before time t).

As shown in Fig. 2, the accumulated release amount of puerarin through the rabbit cornea *in vitro* (Q) presented a linear correlation with permeating time (t). Apparent permeability coefficient (p_{app}, cm /s) can be calculated by the formula:

$$p_{app}=\frac{\Delta Q}{\Delta t \cdot C_0 \cdot A \cdot 60}$$

(C_0: the drug concentration in the donor chamber, A: significant area of the cornea, dQ/dt: the slope rate of the steady state in Q-t curve). The flow rate of the steady state J_{ss} can be calculated by the formula: $J_{ss}=C_0 \cdot p_{app}$.

As shown in Table 1, compared with the p_{app} and J_{ss} of puerarin with 4% (w/v) PVP, those of puerarin with 5% HP-β-CD had exceedingly significant differences ($p<0.01$) and those of puerarin with 10% HP-β-CD had significant differences ($p<0.05$). But there were no significant differences ($p>0.05$) between the p_{app} and J_{ss} of puerarin with 4% PVP and those of puerarin with 15% (w/v) HP-β-CD. The p_{app} and J_{ss} of puerarin decreased as the concentration of HP-β-CD increased. No significant changes were observed exiting in the lag time after HP-β-CD was used as substitute for PVP ($p>0.05$).

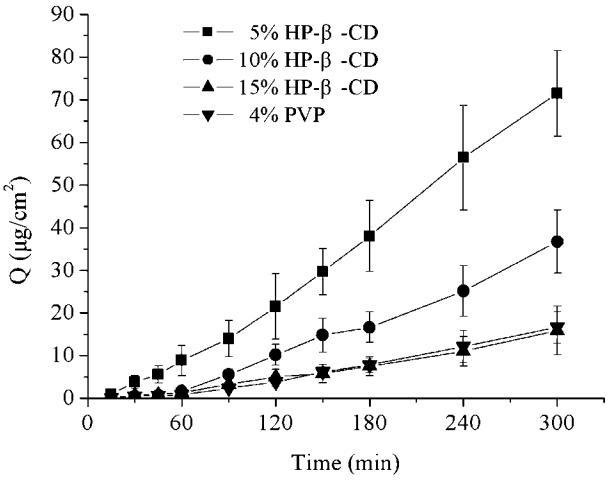

Fig. 2. Permeability of Puerarin across Excised Rabbit Corneas

Each point represents the mean±S.D. ($n=4$).

This experimental result is consistent with the mechanism introduced by Loftssona.[13] In the case of aqueous eye drop solutions, CDs act as true carriers by keeping the hydrophobic drug molecules in solution and deliver them through the aqueous mucin layer to the surface of the ocular barrier (*i.e.*, cornea or conjunctiva) where they partition into the barrier. At low CD concentrations, when there is no excess of CD (*i.e.*, when the donor phase consists of a drug suspension), the permeability coefficient increases with increasing CD concentration. Under these conditions the donor phase is saturated with the drug and, thus, the thermodynamic activity of the drug is at its maximum (*i.e.*, the drug has maximum tendency to partition into the ocular barrier). The amount of dissolved drug increases with increasing CD concentration which results in increased drug diffusion (as drug/CD complex) to the surface of the cornea. At high CD concentrations, when excess CD is present (*i.e.*, when the donor phase consists of a drug solution), the permeability coefficient decreases with increasing CD concentration due to decreased drug activity in the donor phase. In this experiment, when the HP-β-CD concentration exceeds 5%, there might be excess of HP-β-CD in the solution. Therefore, as the concentration of HP-β-CD increases, the thermodynamic activity of puerarin decreases, which leads to the decrease of permeability of puerarin as well.

The corneal hydration value (normally between 76 and 80%) is an important factor in evaluating corneal tissue irritability *in vitro*. If the hydration value exceeds 83%, it indicates that the cornea has suffered some damage.[18] As shown in Table 2, the hydration value from fresh corneas was measured as 78.31±1.24%. The values of all the test groups increased slightly from those of fresh corneas, but there was no

Table 1. *In Vitro* Penetration Parameters of Rabbit Corneas

Groups	Q-t regression equation	r	$J_{ss}\times10^3$ (mg/cm²·s)	$p_{app}\times10^7$ (cm/s)	Lag time (min)
5% HP-β-CD	$Q=0.215t-0.9627$	0.9911	4.51**±0.46	4.78**±0.49	4.5±2.0
10% HP-β-CD	$Q=0.1199t-1.33$	0.9916	2.51*±0.38	2.70*±0.41	11.0±3.8
15% HP-β-CD	$Q=0.068t-0.4294$	0.9881	1.43±0.20	1.53±0.21	6.3±1.8
4% PVP	$Q=0.070t-0.3699$	0.9941	1.46±0.14	1.67±0.16	5.3±2.4

* $p<0.05$ vs. 4% PVP, ** $p<0.01$ vs. 4% PVP. Each value represents the mean±S.D. of four determinations.

significant difference between them ($p>0.05$) and none of them exceeded 83%. From these experimental results, we can see that 5% HP-β-CD can not only improve the solubility of puerarin, but also has better ability to enhance the corneal permeability of puerarin when compared with 4% PVP, 10% and 15% HP-β-CD. So 5% HP-β-CD was adopted as solubilizer in the preparation of *in situ* gelling systems.

Preparation of *in Situ* Gelling Systems Carbopol® 980NF and HPMC E4M were used as gel matrix in the experiments. In our early experiments, we found that compared with other Carbopol® reagents such as 940NF and 974P NF, the viscosity and the transparency of 980NF were optimal. 0.4% (w/v) Carbopol® 980NF has the best ability to form droplets easily when poured from a beaker, which makes administration convenient and dosage accurate and shift into gel in physiological condition (pH 7.4 and 34℃) available, but the pH of 0.4% Carbopol® 980NF solution is only 3.2. So the HPMC E4M was considered to be incorporated into the formulation to keep the viscosity of the gel under physiological condition (pH 7.4 and 34℃) after the concentration of Carbopol® 980NF was reduced, which might increase the pH value of the solution.

As shown by Table 3, the formulations 1—6 at pH5.3±0.2 and the formulations 7—9 at both pH 4.8 ±0.2 and pH 5.3±0.2 had comparatively large viscosity, which leads to bad flowability. The formulations 3, 6—9 at pH 4.3±0.2 were translucent and contained slight white floccule. It was concluded that the branched chain of Carbopol® 980NF did not spread out completely for the lower pH value. Other formulations were transparent. Taking these together, we can see that the formulations 1—2, 4—5 at pH 4.3±0.2 and the formulations 1—6 at pH 4.8± 0.2 made a better performance in both viscosity and transparency. However, considering the fact that the lower the pH value, the lower compliance the patients might have, the formulations 1—6 at pH 4.8±0.2 were chosen for the determination of gelling capacity.

As can be seen from Table 4, the formulations 1, 2 and 4 had more suitable gelling capacity, which completed the gelation immediately and remained for few hours, compared with the formulations 3, 5 and 6, which also completed the gelation immediately, but remained for an extended period. These also can be reflected in the viscosity: compared with the formulations 1, 2 and 4, the formulations 3, 5 and 6 had larger viscosity (>6500 cp), which would cause that the gel was difficult to spread out on cornea and made vision blurring.

Rheological Studies Since the ocular shear rate is very high, ranging from 0.03 1/s during inter-blinking periods to 4250—28,500 1/s during blinking,[22] viscoelastic fluid with a viscosity that is high under

Table 2. Corneal Hydration Level

Groups	Hydration level (%)
Fresh cornea	78.31±1.24
5% HP-β-CD	$78.84^{N.S.}$±0.83
10% HP-β-CD	$81.25^{N.S.}$±1.28
15% HP-β-CD	$79.92^{N.S.}$±0.93
4% PVP	$79.14^{N.S.}$±1.24

Each value represents the mean±S.D. of four determinations.

Table 3. The Viscosity of Gel Formulations at Different pH

Formulation	Carbopol/HPMC*	Viscosity (cp)**		
		pH 4.3±0.2	pH 4.8±0.2	pH 5.3±0.2
1	0.1 : 0.2	43.2±7.6[b]	196.7±17.0[b]	536.4±12.3[b]
2	0.2 : 0.2	68.4±5.8[b]	233.3±12.5[b]	579.5±13.4[b]
3	0.3 : 0.2	94.1±6.5[a]	256.7±12.5[b]	670.9±10.7[b]
4	0.1 : 0.4	96.5±7.0[b]	260.0±16.3[b]	960.3±12.8[b]
5	0.2 : 0.4	126.0±10.4[b]	336.7±9.4[b]	1083.6±9.1[b]
6	0.3 : 0.4	145.1±8.4[a]	390.0±16.3[b]	1204.2±13.4[b]
7	0.1 : 0.6	153.8±10.7[a]	653.3±17.0[b]	1337.6±12.5[b]
8	0.2 : 0.6	316.7±12.3[a]	716.7±4.7[b]	1425.9±11.7[b]
9	0.3 : 0.6	403.4±11.8[a]	773.3±20.6[b]	1603.4±15.6[b]

* It is the rate of the w/v concentrations of Carbopol® 980NF and HPMC E4M. ** The viscosity in all formulations was tested at 12 r/min and 25℃. Each value represents the mean+S.D. ($n=3$). *a*) translucent, *b*) transparent.

Table 4. The Gelling Capacity and Viscosity of Test Formulations at pH 7.4±0.2 and 34℃

Formulation	Carbopol /HPMC*	Viscosity (cp) (pH 7.4±0.2, 34℃)**	Gelling capacity***
1	0.1 : 0.2	3060.0±115.2	++
2	0.2 : 0.2	4173.3±77.2	++
3	0.3 : 0.2	7296.7±87.3	+++
4	0.1 : 0.4	3903.3±102.7	++
5	0.2 : 0.4	6513.3±126.6	+++
6	0.3 : 0.4	8250.0±49.0	+++

Each point represents the mean±S.D. (n=3). * It is the rate of the w/v concentrations of Carbopol® 980NF and HPMC E4M. ** Solutions tested at 12 r/min. *** −: No gelation, +: Gels after a few minutes, dissolves rapidly, ++: Gelation immediate, remains for few hours, +++: Gelation immediate, remains for extended period.

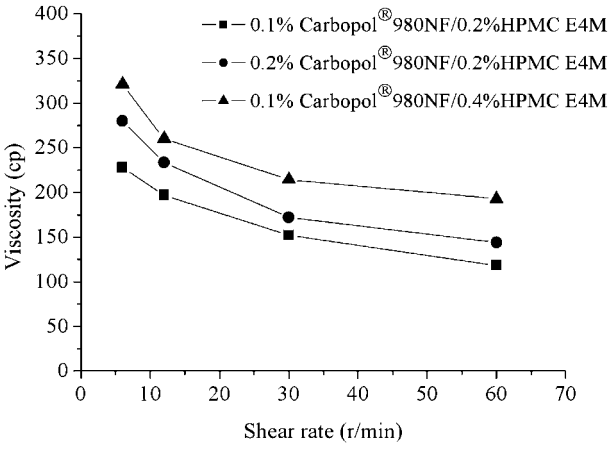

Fig. 3. The Rheological Profiles of Carbopol® 980-HPMC E4M Solution at 25℃ and pH 4.8±0.2

All the measurements were performed in triplicate and the standard deviations were all within 3%.

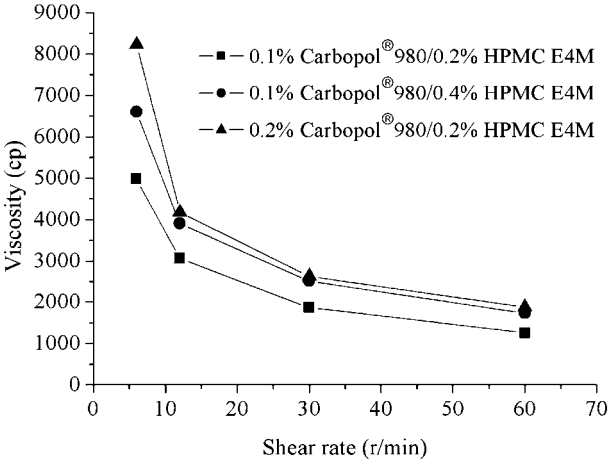

Fig. 4. The Rheological Profiles of Carbopol® 980NF-HPMC E4M Solution at 34℃ and pH 7.4±0.2

All the measurements were performed in triplicate and the standard deviations were all within 3%.

low shear rate condition and low under the high shear rate condition, which is called pseudoplastic fluid, is often preferred, so the dynamic viscosity of formulations 1, 2 and 4 were measured as the change of shear rate under non-physiological condition (pH 4.8±0.2 and 25℃) and physiological condition (pH 7.4 and 34℃), respectively, to investigate the rheology of these formulations. As shown by Figs. 3 and 4, the viscosity of these three formulations all decreased as the shear rate increased, which showed the character of pseudoplastic fluid. During blinking the shearing force on the preparation is large. If the viscosity at high shear rate is too high, this will result in irritation. On the other hand, if the viscosity is too low, it will give rise to increased drainage. The pseudoplastic property of these formulations is in favor of sustaining drainage of drugs from the conjunctiva sac of the eye, simultaneously without blinking difficulty for undergoing shear thinning.

***In Vitro* Release Studies** Figure 5 illustrates the cumulative amount of puerarin released versus the time profiles for formulations 1, 2 and 4, and puerarin eye drops which use 4% PVP as a solubilizer. As shown in this figure for the puerarin eye drops is that almost all of the puerarin released immediately after the start of the release experiment. The drug released about 83.0% to the medium after 1 h. For formulation 1, the drug released roughly 39.6% to the medium after 1 h and approximately 89.8% released after 6 h. It obviously exhibited some delayed release effects. In formulation 2, puerarin released roughly 38.7% to the medium after 1 h and approximately 80.7% released after 6 h. Thus, formulation 2 has a better delayed release effect. Formulation 4 exhibited a slightly improved release effect than formulation 2, as the drug released about 37.3% to the medium after 1 h and approximately 79.4% of puerarin released after 6 h. The reason that the delayed release effect of formulation 1 is worse than those of formulations 2 and 4 may be ascribed to its lower viscosity. The viscosity and the delayed release effect of the formulations 2 and 4 is close to each other, but considering that the less the usage amount of Carbopol® 980 is, the less the potential stimulus to the eye will be, we adopted formulation 4 containing 0.1% (w/v) Carbopol® 980 and 0.4% (w/v) HPMC E4M as the optimal formulation.

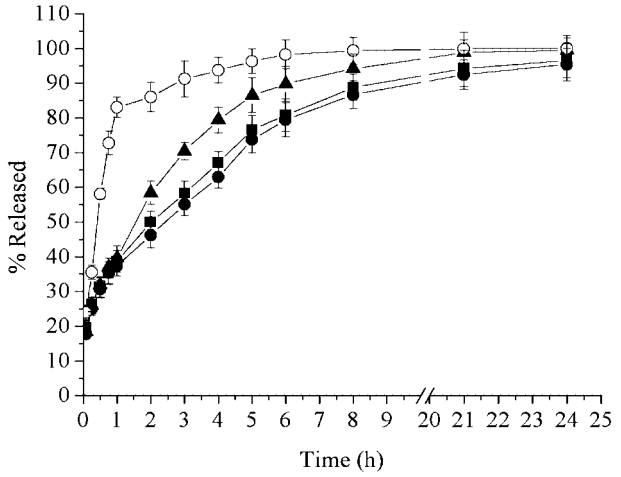

Fig. 5. Cumulative Amount of Puerarin Released from Carbopol® 980NF-HPMC E4M and Control (Puerarin Eye Drops-containing 40 g/LPVP)

(▲): 0.1% Carbopol® 980/0.2% HPMC E4M, (■): 0.1% Carbopol® 980/0.4% HPMC E4M, (●): 0.2% Carbopol® 980/0.2% HPMC E4M, (○): puerarin eye drops-containing 40 g/LPVP. Each point represents the mean±S.D. (n=3).

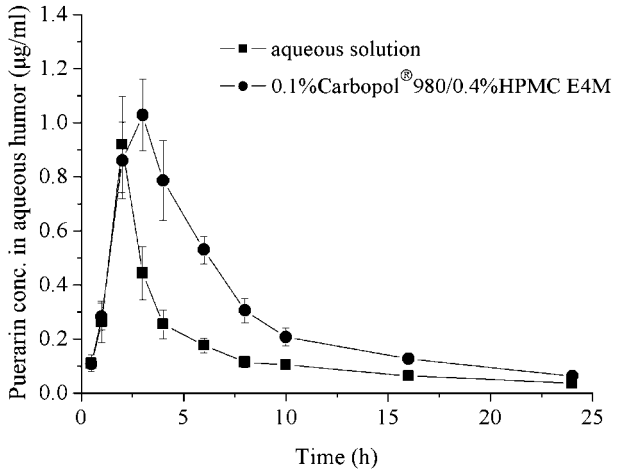

Fig. 6. Puerarin Concentrations in Rabbit Aqueous Humors at Different Time after Topical Application of the Puerarin Eye Drops-containing 4% PVP and the Puerarin *In situ*-forming Gel Containing 0.1% Carbopol® 980 and 0.4% HPMC E4M in the Rabbit Eyes

Each point represents the means±S.D. (n=4).

***In Vivo* Study** Figure 6 shows the level of puerarin in aqueous humor after instillation of 50 μl of puerarin formulated as aqueous solution and as pH-triggered *in situ* gel containing 0.1% Carbopol® 980 and 0.4% HPMC E4M, and the corresponding kinetic parameters are summarized in Table 5. Student's t-test was used to statistically compare the results obtained with the two different formulations. The aqueous humor content of puerarin was significantly higher ($p<0.01$), at 3, 4, 6, 8, 10 h, after administration of puerarin pH-triggered *in situ* gel formulations than that obtained after instillation of puerarin eye drops. In comparison with aqueous solution, the *in situ* gel exhibited 1.76-fold and 2.17-fold greater the time required to reach maximum concentration (T_{max}) and the area under the curve in 24 h ($AUC_{0-24\,h}$), respectively. But the maximum level of puerarin in aqueous humor (C_{max}) exhibited no significant difference ($p>0.05$) between the aqueous solution and *in situ* gel. Although the difference between the constant of absorption rate (K_a) of the aqueous solution and *in situ* gel has no statistical significance ($p>0.05$), the $t_{1/2}$(ka) of *in situ* gel exhibited a 1.82-fold greater than that of aqueous solution. In the elimination stage, the constant of elimination rate (K_e) of *in situ* gel has reduced significantly compared with that of aqueous solution ($p<0.01$). The $t_{1/2}$(ke) of *in situ* gel has increased 1.62-fold in comparison with that of aqueous solution. These suggest that with the use of *in situ* gel, more amount of drug could be absorbed into the eye before it was washed out of the conjunctiva sac by tears, and the time for the elimination of puerarin from the aqueous humor was consequently prolonged.

Table 5. Pharmacokinetic Parameters of Puerarin in Aqueous Humor

Parameters	Aqueous solution	*In situ* gel
C_{max} (μg/ml)	0.63±0.16	0.81±0.12
T_{max} (h)	1.35±0.23	2.38±0.26**
$AUC_{0-24\,h}$ (μg*h/ml)	2.65±0.65	5.76±0.70**
K_a (1/h)	1.27±0.57	0.65±0.13
K_e (1/h)	0.43±0.07	0.26±0.02**
$t_{1/2}$(ka) (h)	0.61±0.19	1.11±0.23*
$t_{1/2}$(ke) (h)	1.64±0.27	2.66±0.25**

Note: Each point represents the mean±S.D. (n=4). Aqueous solution contains 4% PVP and *in situ* gel contains 0.1% Carbopol® 980 and 0.4% HPMC E4M. C_{max}: maximum aqueous humor concentration, T_{max}: time of reach C_{max}, $AUC_{0-24\,h}$: area under the aqueous humor level curve between time 0 and 24 h. K_a: constant of absorption rate, K_e: constant of elimination rate, $t_{1/2}$(ka): half-life of the absorption phase, $t_{1/2}$(ke): half-life of the elimination phase. Statistically significant difference from the aqueous solution at the level of * $p<0.05$ and ** $p<0.01$.

CONCLUSIONS

In the present study, we found that HP-β-CD could increase the solubility of puerarin and 5% HP-β-CD enhanced its ocular permeability significantly. The optimum concentrations of Carbopol® 980NF and HPMC E4M for use as a pH-triggered *in situ* gelling

vehicle for ophthalmic drug delivery were 0.1% and 0.4%, respectively. When these two vehicles were combined, the gel strength and the gelling capacity under physiological condition were appropriate. This combined solution could flow freely under non-physiological condition and showed the character of pseudoplastic fluid under both conditions. Both *in vitro* and *in vivo* results indicated that the combined polymer systems performed better in retaining puerarin than puerarin eye drops did. Therefore, it is a viable alternative to conventional puerarin eye drops by virtue of its abilities that it can not only be readily administered and decrease the frequency of administration, thus resulting in better patient acceptance, but also prolong the precorneal residence time to get higher bioavailability and reduce the systemic side-effects caused by the drainage from the nasolacrymal duct.

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