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Promoting effect of borneol on the permeability of puerarin eye drops and timolol maleate eye drops through the cornea *in vitro*

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Studies on the influence of borneol on the penetration of puerarin eye drops and timolol maleate eye drops through the cornea, and evaluation of the ocular irritability were conducted to provide a theoretical basis for the application of borneol in enhancing corneal permeability. The cornea penetrative experiment *in vitro* was conducted to observe the quantitative change of puerarin and timolol maleate penetrated through the cornea after administering different dosages of borneol. The corneal hydration level and blinking frequency were recorded as irritability indexes *in vitro* and *in vivo*. The steady-flow J of high, middle and low dosage groups of puerarin eye drops with borneol were increased by 49%, 32%, 5% respectively, and permeability parameter Kp increased by 49%, 32%, 5% respectively, as compared to that of the control group. The steady-flow J of high dosage group of timolol maleate eye drops with borneol was increased by 5%; middle and low dosage groups with borneol were decreased by 6%, 3% respectively. The permeability parameter Kp of high dosage group increased by 5%, while middle and low dosage groups with borneol were decreased by 6%, 3% respectively, as compared to that of the control group. Evaluation showed no ocular irritability caused by borneol. The results of this study suggest that the promoting effect of borneol on the permeability of drugs through the cornea *in vitro* is selective, which indicates that borneol has the potential to be used as an ophthalmic penetration enhancer.

1. Introduction

The common problems related to the ocular administration include short lasting time of the medication, difficulty for medication to penetrate the cornea, and local irritability when applied improperly (Saettone 2002). The bioavailability of eye drops is very low, usually less than 10%, thus it is difficult to achieve a therapeutic effect (Lee and Robinson 1986; Tang et al. 1987). In the past three decades, many efforts have been made to improve the topical bioavailability of ophthalmic drugs. Researchers are focusing on the following aspects: (1) to increase the stickiness of pharmaceutical preparations, thus prolonging the lasting time of the medication in the ocular area (Romanelli et al. 1994; Nomura et al. 1994); (2) to use pro-drugs with better penetrability, thus improving the penetrability of the drug itself (Suhonen et al. 1995; Jarvinen et al. 1995); (3) to apply penetration enhancers, thus increasing the permeability of the cornea (Di et al. 2004; Sasaki et al. 1999; Tang et al. 1994; Montenegro et al. 2003). As regard to the use of penetration enhancers, extensive investigations have been conducted in the transdermal-drug delivery system. For instance, azone was proved to promote transdermal penetration of both lipophilic and hydrophilic drugs. Nowadays these compounds, such as azone, dimethyl sulfoxide (DMSO), decamethonium, glycocholate, and cholate, are also studied as ocular penetration enhancers in the laboratory. The corneal epithelium and the keratoderma are different in cellular type, pharmakinetic and metabolic processes; meanwhile the cornea is rather sensitive to a chemical stimulus of foreign materials. Therefore, these penetration enhancers will easily generate irritability and toxicity, and the applicable concentrations are generally lower than those used on the skin. Taking azone as an example, 0.1% or a slightly higher concentration can cause ocular hypersensitivity, discomfort or toxicity (Durand-Cavagna et al. 1989). Ocular tolerance of nine potential absorption promoters has been investigated by Furrer et al (2002). The results indicate that DMSO, decamethonium, Tween 20, Brij 35, EDTA, glycocholate, and cholate cause less than 16% of the corneal surface damaged. With sodium fusidate and saponin, more than 30% of the cornea was injured. At present, these penetration enhancers are still under study without any breakthrough in the researches. There are no ophthalmic preparations containing these penetration enhancers commercially available.

In China, traditional Chinese medicine (TCM) is widely used in the treatment and prevention of eye diseases. In ophthalmology, borneol is one of the frequently used traditional Chinese medicinals, recorded in the Pharmacopoeia of the People's Republic of China, Edition 2005,

Vol. 1. In the theory of TCM, it has the action of restoring consciousness, clearing heat, and relieving pain. In China, it has been used in the clinic for almost a thousand year. In China's earliest ophthalmography "Secret Records of Ophthalmology (mi chuan yan ke long mu lun)", 24 external prescriptions for the treatment of 74 syndromes are recorded. Among these, borneol was used in 15 prescriptions. Some of the prescriptions reflect the approach of curing internal diseases by external treatment. Statistical data indicates that 55% of ophthalmic external prescriptions in the ancient ophthalmographies contain borneol, such as in "Essence of the Sliver Sea (ying hai jing wei)", "Scrutiny of the Priceless Jade Case (shen shi yao han)", "Compendium of Ophthalmology (mu jing da cheng)", (Fan et al. 1998). In China's "National Collection of Chinese Medicine Products (quan guo zhong cheng yao chu fang ji)", there are 76 ophthalmic external prescriptions, 73% of which containing borneol. Borneol is also commonly used in modern TCM ophthalmic preparations, such as: Pearl Eye Drops (zhen zhu ming mu di yan ye), Eight Treasures Eye Powder (ba bao yan yao), Mayinglong Eight Treasures Eye Ointment (ma ying long ba bao yan gao), etc. But in TCM, borneol is not used as the medicinal principle; it is mainly used as an assistant or a messenger in the prescriptions. As "Extension of the Materia Medica (ben cao yan yi)" recorded that "borneol is not potent enough when used alone; when it is used as an assistant or a messenger, borneol is effective." "Borneol is aromatic with a moving nature; it can guide other medicinals going upwards" (Kou 1990). Modern researches indicated that the role of borneol as an assistant or a messenger in the prescriptions was related to its action to promote penetration and to reinforce the action of other ingredients in a formula. Research efforts were focusing on the aspects of borneol to promote other medicinals penetrating the blood-brain barrier (BBB) (Dong et al. 2002; Liu et al. 1994; Chen and Wang 2004) and the skin membrana mucosa (Gao et al. 2004; Xu and Wang 2001), although preliminary research was conducted to observe its action to promote ocular penetration. Researchers investigated the mechanism of the action of borneol to promote penetration by means of electronic spin resonance (ESR) at inframolecular level. Cultivated corneal epithelium was divided into the experimental group and two control groups. The characteristic sequence of the phospholipid molecule in the cell membrane of the corneal epithelium was determined by ESR. And results indicate the promoted osmosis of corneal epithelium by borneol was associated with the characteristic sequence of the phospholipid bimolecule in the cell membrane of corneal epithelium (Fan et al. 1998; Fan et al. 2003).

However, in the clinical practice of TCM ophthalmology, ocular irritability occurred because of improper dosage and administration. Some people even considered that borneol might be somehow toxic.

Taking puerarin eye drops and timolol maleate eye drops as the model, the influence of borneol on the penetration through the cornea *in vitro*, and an evaluation on the ocular irritability in rabbits are discussed in detail in this paper.

2. Investigations and results

2.1. Penetrative experiments in vitro

The accumulated release amount of medicine passing through a cornea was calculated by the following formula:

$$Q = C_n + V_0/V \sum_{s=1}^{s=n-1} Cps \qquad (1)$$

(Q: the accumulated release amount within time t; C_n: the measured value of concentration within time t; V: the total volume of releasing liquid; V_0: sampling volume each time; Cps: the measured value of concentration before time t)

The accumulated release amount of different puerarin eye drops containing no borneol, 0.025%, 0.05% and 0.1% borneol through the rabbit's cornea *in vitro* within different times is listed in Table 1.

As showed in Table 1, the accumulation penetration of rabbit's cornea *in vitro* (Q) presented a linear correlation with permeating time (t). Equations can be fit as one-element equation of linear regression. The homeostasis flow rate J (amount of medicine through corneal within unit area and unit time) can be calculated by the slope rate of $Q - t$.

$$J = V/A \cdot dQ/dt \qquad (2)$$

(V: total volume of releasing liquid; A: significant area of corneal; dQ/dt: slope rate of homeostasis). Permeability coefficient Kp can be calculated by

$$Kp = J/(VC_0) \qquad (3)$$

(J: homeostasis flow rate of medicine; V: total volume of releasing liquid; C_0: medicine concentration in administering cell).

As shown in Table 2, homeostasis flow rates J of high-dose group, middle-dose group and low-dose group which contain borneol increased by 49%, 32%, 5%, and permeability coefficient Kp increased accordingly, comparing to that of blank group, indicating that borneol can promote puerarin to permeate cornea *in vitro*.

The accumulated release amount of different timolol maleate drops containing no borneol, 0.025%, 0.05% and 0.1% borneol through rabbit's cornea *in vitro* within different time is shown in Table 3.

Table 1: Accumulated release amount of different puerarin eye drops through rabbit's cornea *in vitro* (mean ± S.D.)

Groups	Time (h)										
	0.25	0.50	0.75	1.00	1.50	2.00	2.50	3.00	4.00	5.00	6.00
Blank	3.60 ± 0.50	4.34 ± 0.25	4.87 ± 0.16	5.58 ± 0.22	8.08 ± 0.27	11.29 ± 1.94	14.82 ± 1.50	19.56 ± 2.08	24.99 ± 1.78	29.95 ± 3.90	36.52 ± 4.73
Low	4.42 ± 0.64	6.83 ± 1.38	8.35 ± 1.86	10.38 ± 3.19	12.87 ± 2.50	16.62 ± 3.25	21.95 ± 3.79	24.90 ± 2.86	31.01 ± 3.38	35.20 ± 5.05	39.66 ± 4.97
Middle	5.37 ± 1.51	7.70 ± 2.21	8.95 ± 2.94	10.89 ± 4.28	13.54 ± 5.61	17.14 ± 6.57	21.36 ± 5.70	27.06 ± 5.01	33.73 ± 4.08	41.25 ± 3.75	51.77 ± 5.69
High	5.10 ± 1.15	8.86 ± 0.25	10.31 ± 0.52	15.30 ± 0.41	18.99 ± 0.77	22.64 ± 0.43	26.61 ± 1.40	31.72 ± 1.32	37.42 ± 0.88	47.15 ± 1.95	59.96 ± 2.78

Table 2: Penetration parameters of different puerarin eye drops through rabbit's cornea *in vitro*

Groups	Q-t Regression equation	r	J ($\mu g \cdot cm^{-2} \cdot h^{-1}$)	Kp ($cm^{-2} \cdot h^{-1}$)
Blank	$Q = 5.9509\ t + 0.5350$	0.9963	30.32	3.03×10^{-3}
Low	$Q = 6.2986\ t + 4.1164$	0.9937	32.09	3.21×10^{-3}
Middle	$Q = 7.8984\ t + 2.6768$	0.9977	40.25	4.02×10^{-3}
High	$Q = 8.8976\ t + 4.3881$	0.9960	45.34	4.53×10^{-3}

Table 3: Accumulated release of different timolol maleate drops through rabbit's cornea *in vitro* (mean ± S.D.)

Groups	Time (h)										
	0.25	0.50	0.75	1.00	1.50	2.00	2.50	3.00	4.00	5.00	6.00
Blank	1.18 ± 1.34	2.82 ± 2.38	5.39 ± 3.27	7.55 ± 3.97	12.14 ± 5.60	16.96 ± 7.31	21.46 ± 8.69	25.63 ± 10.19	34.06 ± 12.16	34.46 ± 9.48	52.59 ± 16.41
Low	1.19 ± 1.15	2.59 ± 0.98	5.20 ± 1.38	7.16 ± 1.55	12.32 ± 1.30	16.83 ± 2.61	21.10 ± 3.92	25.36 ± 3.90	32.50 ± 4.93	39.24 ± 7.28	46.68 ± 8.09
Middle	0.63 ± 0.52	2.04 ± 0.92	3.93 ± 1.36	5.90 ± 1.91	10.05 ± 3.15	13.90 ± 4.25	17.54 ± 5.42	21.32 ± 6.85	28.51 ± 10.14	42.17 ± 12.79	41.57 ± 14.33
High	0.65 ± 0.55	2.30 ± 0.99	4.68 ± 1.49	7.31 ± 1.94	12.37 ± 3.13	16.71 ± 4.00	21.20 ± 4.75	25.49 ± 5.53	33.61 ± 6.97	41.93 ± 8.87	50.93 ± 10.86

Table 4: Penetration parameters of different timolol maleate drops through rabbit's cornea *in vitro*

Groups	Q-t Regression equation	r	J ($\mu g \cdot cm^{-2} \cdot h^{-1}$)	Kp ($cm^{-2} \cdot h^{-1}$)
Blank	$Q = 8.3189\ t - 0.5647$	0.9883	42.39	8.48×10^{-3}
Low	$Q = 8.0185\ t - 0.1701$	0.9979	40.86	8.17×10^{-3}
Middle	$Q = 7.7686\ t - 1.6479$	0.9919	39.59	7.92×10^{-3}
High	$Q = 8.7601\ t - 1.3602$	0.9995	44.64	8.93×10^{-3}

As showed in the Table, the accumulation penetration of rabbit's cornea *in vitro* (Q) presented linear correlation with permeating time (t). Equations can be fit as one-element equation of linear regression. The homeostasis J and Kp can be calculated using Eqs. (2) and (3).

As showed in Table 4, homeostasis flow rates J of high-dose group containing borneol increased by 5%; the permeability coefficient Kp increased by 5%, while in the middle-dose group and the low-dose group it decreased by 6% and 3%; the permeability coefficient Kp decreased a accordingly, comparing to that of blank group.

2.2. Evaluation of irritability

The hydration value of the cornea is an important index to evaluate tissue irritability *in vitro*. If the hydration value exceeds 83%, it can be judged that the cornea has suffered from certain damage, as the normal hydration value of the cornea is between 76% and 80% (Table 5).

The hydration value of fresh peeled cornea was measured as 77 ± 1%. The values of all the test groups showed no significant difference from that of the control group ($P > 0.05$), after a 6 hour permeation experiment.

There was no significant difference betweeen the test groups and the control group ($P > 0.05$).

Table 5: Cornea hydration level (mean ± S.D., n = 4)

Groups	Puerarin		Timolol maleate	
	Hydration level (%)	Increasing (%)	Hydration level (%)	Increasing (%)
Blank	79.05 ± 1.26	2.25	79.58 ± 0.89	2.94
Low	79.25 ± 1.30	2.51	79.76 ± 1.14	3.17
Middle	79.92 ± 0.95	3.38	80.73 ± 0.64	4.42
High	81.14 ± 0.81	4.95	81.52 ± 1.08	5.45

Table 6: Blinking counts in 5 min after instillation of 25 µl enhancer solution in rabbit eyes (mean ± S.D., n = 4)

Groups	Blank	Low	Middle	High
Control group	3.8 ± 13.3	4.3 ± 11.8	4.5 ± 12.8	4.8 ± 10.5
Test group	4.3 ± 11.8	4.5 ± 22.2	4.8 ± 20.2	5.0 ± 16.3

Comparing the hydration value of the cornea *in vitro* exposed to borneol solutions of different concentrations (Table 5), with irritability *in vivo* (Table 6), we found a linear correlation between both. So, it is considered that borneol applied on the cornea will not cause any irritability.

3. Discussion

According to the China's national standards, the commonly used concentration of borneol is 0.1% in ophthalmic preparations. Therefore, in this paper, the high concentration group was determined as 0.1%. Test groups of other doses were prepared containing 0.025%, 0.05% and 0.1% borneol.

The absorbing degree and release rate of drug into eyes are mainly related to the corneal barrier. They are also related to such physicochemical properties of the drug as liposolubility, molecular size and shape, electric charge and degree of ionization. The corneal barrier consists of the epithelium, stroma and endothelium, which form the three primary layers through which substances can permeate. For water-soluble drugs, the epithelium is the main barrier and the endothelium and stroma have little influence. For many lipophilic drugs, the epithelium has less influence and the endothelium and stroma are the main barriers. It is thus evident from this experiment that the

steady-flow J and the permeability parameter Kp of high, middle and low dosage groups of puerarin eye drops with borneol were all increased and compared to that of the control group. The steady-flow J and the permeability parameter Kp of high dosage group of timolol maleate eye drops with borneol were all slightly increased, while middle and low dosage groups with borneol were all decreased, as compared to that of the control group. So there is no obvious correlation between the permeability of timolol maleate and the concentration of borneol. To make a further deduction from this result, borneol has promoted corneal penetration of puerarin which is lipophilic, while has little influence on the corneal penetration of timolol maleate which is hydrophilic. Considering the property of borneol, which is a lipophilic substance, borneol maybe have some influence on permeability of the stroma.

The current application of borneol is still limited to the experiences of TCM. Some researchers conducted preliminarily studies on the mechanism of action of borneol to promote penetration at an inframolecular level (Fan et al. 1998). Results from this paper show that borneol obviously promotes penetration when used in eye drops without any irritability. This indicates that borneol is a potential ingredient for ophthalmic preparations and is useful for clinical applications.

4. Experimental

4.1. Animals

New Zealand albino rabbits of either sex obtained from the nursery of the Experimental Animal Professional Committee, Sichuan, China, weighing between 2.5 and 3.0 kg, were individually housed in an air-conditioned and light-controlled room at 25 °C ± 1 °C and 70% ± 5% relative humidity. They received a standard pellet diet and water. All animals were healthy and free of clinically observable ocular abnormalities. Animals were separated at random into a control group and low, middle, high dosage groups.

The animals were maintained and handled according to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research. All experiments were approved by the local ethics committees for animal experimentation.

4.2. Instruments and materials

LC-2010A HPLC (Shimadzu, Japan); Sartorius BP211D electron balance (reciprocal sensibility 0.1 mg; 0.01 mg weight limit 210 g; 80 g); TGL-16G high speed hydroextractor; Valia-chien level diffusion cell (Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences); JB-2 constant temperature magnetic stirring apparatus (Shanghai Lei Chi Xin Jing Apparatus Co. Ltd.); precision microliter pipette (Gilson, France). Puerarin eye drops (Batch number: 040427, supplied by Zhejiang Pinghu Shapuaisi Pharmaceutical Co. Ltd., China). Timolol maleate eye drops (Batch number: 04090103, supplied by Wuhan Wujing Pharmaceutical Co. Ltd., China).

Chemical Reference Standards (CRS): Puerarin (110752–200209, for assaying), supplied by National Institute for the Control of Pharmaceutical and Biological Products. Timolol maleate, supplied by Tianjin Central Pharmaceutical Co, Ltd., China.

Glutathione bicarbonate Ringer's Solution (GBR): prepared in two parts. Solution A: containing 12.4 g NaCl, 0.716 g KCl, 0.206 g $NaH_2PO_4 \cdot H_2O$ and 4.908 g $NaHCO_3$ per liter; Solution B: containing 0.23 g $CaCl_2 \cdot 2\,H_2O$, 0.318 g $MgCl_2 \cdot 6\,H_2O$, 1.8 g glucose, and 0.184 g oxidized glutathione per liter. Solution A and B were stored in sub-ambient temperature and mixed before use.

Methanol is chromatographic grade (Fisher Chemicals),water is bi-distilled, other reagents are analytical grades.

4.3. Assaying of puerarin eye drops

4.3.1. Chromatographic system and system suitability

Chromatographic column: Diamonsil C_{18} Column (5 μm, 150 × 4.6 mm); mobile phase: methanol-water : glacial acetic acid (25 : 75 : 1); flow rate: 1.0 ml/min; wavelength of the detector: 250 nm, column temperature: 30 °C; sample size: 20 μl. The number of theoretic plates of the column is not less than 1500, calculated with the reference to the peak of puerarin.

4.3.2. Determination of the wavelength of the detector

Inject each of the reference solution and the test solution, determine under chromatographic condition above, and ultra-violet scan on SPD-M10Avp diode array detector, each of the reference solutions and the test solution of (c, $\mu g \cdot ml^{-1}$) puerarin presents maximum absorption at 250 nm wavelength. 250 nm is selected as the wavelength of the detector.

4.3.3. Standard curve

Dissolve accurately weighed puerarin CRS in methanol, then add newly prepared GBR solution to produce a solution containing 800 μg per ml as the standard solution. Accurately weigh suitable amount of standard solution, add newly prepared GBR solution respectively to prepare a series of reference standard solutions of different concentrations. Inject 20 μl, under the chromatographic condition above, record chromatogram. Taking the concentration of puerarin as X-axis and peak area (A) as Y-axis, conduct linear regression to get the regression equation: $Y = 1.1365 \times 10^{-8}\,X + 2.4486 \times 10^{-3}$, $r = 0.9999$. The result indicated that the linear correlation was good when the concentration of puerarin was between 0.256 $\mu g \cdot ml^{-1}$ and 800 $\mu g \cdot ml^{-1}$.

4.3.4. Recovery rate test

Weigh accurately a suitable amount of standard solution, add newly prepared GBR solution respectively to produce three batches of puerarin standard solution of three different concentrations (high: 160 $\mu g \cdot ml^{-1}$; middle: 32 $\mu g \cdot ml^{-1}$; low: 1.28 $\mu g \cdot ml^{-1}$). Inject accurately 20 μl each of standard solution and the test solutions separately into the column, and measure peak area, calculate recovery amount by external standard method. The ratio between recovery amount and adding amount is the recovery rate. The average recovery is 99.5%, see Table 7.

4.3.5. Precision and reproducibility

Prepare puerarin standard solution of three different concentrations (1.28 $\mu g \cdot ml^{-1}$, 32 $\mu g \cdot ml^{-1}$, 160 $\mu g \cdot ml^{-1}$). Each solution was determined five times within a day. The relative standard deviation (RSD) values of three different concentrations were calculated to obtain the intra-day precision. The above-mentioned standard solution of three different concentrations was then determined once a day for continuously five days to obtain the inter-day reproducibility, see Table 8.

4.4. Assaying of timolol maleate eye drops

4.4.1. Chromatographic system and system suitability

Chromatographic column: kromasil C_{18} Column (5 μm, 150 × 4.6 mm); mobile phase: methanol-water : triethylamine (40 : 60 : 0.1 pH = 5); flow rate: 1.0 ml/min; wavelength of the detector: 295 nm, column temperature: 25 °C; sample size: 20 μl. The number of theoretic plates of the column is not less than 2000, calculated with the reference to the peak of timolol maleate.

Table 7: Recovery rate of puerarin from GBR solution

Group	No.	Amount added ($\mu g \cdot ml^{-1}$)	Amount observed ($\mu g \cdot ml^{-1}$)	Recovery (%)	Average recovery (%)	RSD (%)
	1	139.63	142.00	101.7		
High	2	139.63	140.14	100.4		
	3	139.63	142.26	101.9		
	4	27.87	27.61	99.1		
Middle	5	27.87	28.30	101.5	99.5	3.17
	6	27.87	28.98	104.0		
	7	1.48	1.43	96.6		
Low	8	1.48	1.41	95.3		
	9	1.48	1.41	95.3		

Table 8: Intra-day precision and inter-day reproducibility of puerarin (n = 5)

Concentration ($\mu g \cdot ml^{-1}$)	Intra-day		Inter-day	
	Peak area	RSD (%)	Peak area	RSD (%)
1.28	137717.0	1.1	136684.0	2.2
32	2762490.0	0.8	2777287.0	0.9
160	14055778.0	0.4	14027184.7	0.6

4.4.2. Determination of the wavelength of the detector

Inject each of the reference solution and the test solution, determine under chromatographic condition above, and ultra-violet scan on SPD-M10Avp diode array detector, each of the reference solution and the test solution of (c, $\mu g \cdot ml^{-1}$) timolol maleate presents a maximum absorption at 295 nm wavelength. 295 nm is selected as the wavelength of the detector.

4.4.3. Standard curve

Dissolve accurately weighed timolol maleate CRS (dried at 105 °C to a constant weight) in newly prepared GBR solution to produce a solution containing 400 μg per ml as the standard solution. Accurately weigh a suitable amount of standard solution, add newly prepared GBR solution to prepare a series of reference standard solutions of different concentrations. Inject 20 μl, under the chromatographic conditions above, record chromatogram. Taking the concentration of timolol maleate as X-axis and peak area (A) as Y-axis, conduct linear regression to get regression equation: $Y = 24129\ X + 33059$, $r = 0.9999$. The result indicated that the linear correlation was good when the concentration of timolol maleate was between 0.128 $\mu g \cdot ml^{-1}$ and 400 $\mu g \cdot ml^{-1}$.

4.4.4. Recovery rate test

Weigh accurately a suitable amount of standard solution, add newly prepared GBR solution respectively to produce three batches of timolol maleate standard solution of three different concentrations (high: 80 $\mu g \cdot ml^{-1}$; middle: 16 $\mu g \cdot ml^{-1}$; low: 0.64 $\mu g \cdot ml^{-1}$). Inject accurately 20 μl each of standard solution and the test solution separately into the column, and measure peak area, calculate recovery amount by external standard method. The ratio between recovery amount and adding amount is the recovery rate. The average recovery is 100.1%, see Table 9.

4.4.5. Precision and reproducibility

Prepare timolol maleate standard solution of three different concentrations (0.64 $\mu g \cdot ml^{-1}$, 16 $\mu g \cdot ml^{-1}$, 80 $\mu g \cdot ml^{-1}$). Each solution was determined five times within a day. The relative standard deviation (RSD) values of three different concentrations were calculated to obtain the intra-day precision. The above-mentioned standard solution of three different concentrations was then determined once a day for continuously five days to obtain the inter-day reproducibility, see Table 10.

4.5. Penetration experiments in vitro

4.5.1. Preparation of the isolated cornea

Rabbits which were healthy and free of clinically observable ocular abnormalities were euthanised with an intravenous lethal dose of sodium pentobarbital (Beijing Chemical Reagents Co., Ltd., China). The whole eyes were enucleated from their sockets and the corneas with a 2 mm ring of sclera were immediately excised in accordance with the procedure described elsewhere. Various tissues of the eye were dissected leaving the corneas, which were then washed with distilled water, and preserved in newly prepared GBR solution. The penetrative experiments in vitro must be started within 20 mins.

4.5.2. Medium release

Newly prepared GBR solution was used (Rojanasakul et al. 1989).

4.5.3. Penetration experiments

The experiments were performed according to the methods reported elsewhere (Montenegro et al. 2003; Wang et al. 2002). Vilia-Chien perfusion chambers were used, which include donor chamber and reservoir chamber. The effective penetrative area is about 0.795 cm^2. 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. Magnetic stirring bars were put in donor chamber and reservoir chamber. The isolated cornea was placed and clamped between two compartments of the perfusion chambers, and the epidermis faces the donor chamber.

Penetration experiments of puerarin eye drops: For the control group, 2.8 ml GBR solution, 1 ml puerarin eye drops, and 0.2 ml propylene glycol were added to the donor chamber. 4 ml GBR solution was added to reservoir chamber. For three experimental groups, 2.8 ml GBR solution, 1 ml puerarin eye drops, and 0.2 ml 0.5%, 0.25%, 0.125% borneol in propylene glycol solution were respectively added to the donor chamber (which is equivalent to adding puerarin eye drops of containing no borneol, 0.025%, 0.05% and 0.1% borneol).

Penetration experiments of timolol maleate eye drops: For the control group, 2.8 ml GBR solution, 1 ml timolol maleate eye drops, and 0.2 ml propylene glycol were added to the donor chamber. 4 ml GBR solution was added to the reservoir chamber. For three experimental groups, 2.8 ml GBR solution, 1 ml timolol maleate eye drops, and 0.2 ml 0.5%, 0.25%, 0.125% borneol in propylene glycol solution were added to the donor chamber (which is equivalent to adding timolol maleate eye drops of containing no borneol, 0.025%, 0.05% and 0.1% borneol).

The perfusion chambers were placed on the magnetic stirrer, and thermostated by circular constant temperature water (34 ± 0.5 °C). 200 μl solutions were withdrawn from the reservoir chamber at 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0 h from the beginning with a precision micro liter pipette. Each sample was immediately replaced with an equal volume of GBR solution to maintain a constant volume. Centrifugate the sample solution (10000 r/min, 10 mins), inject 20 μl of the supernate and determine. Plot the curve diagram of medicine release *in vitro*, fit equations and calculate.

4.6. Evaluation of ocular irritability

4.6.1. Evaluation of corneal hydration level

Wet corneal weight (Wb) was obtained after careful removed of the scleral tissue; each cornea was then desiccated at 70 °C overnight to give the corresponding dry corneal weight (Wa). The percent corneal hydration level (%HL) was calculated according to:

$$\%HL = (Wb - Wa)/Wb \times 100 \quad (4)$$

4.6.2. Evaluation of ocular irritability in vivo

Phosphoric acid buffer solution (pH 7.4 25 μl) containing penetration enhancers was administrexed in the conjunctiva bursa saccus conjunctivae of rabbits. Rabbits were forced to blink for one time to make sure the solution distributed evenly. The times of blink within 5 min were noted, comparing to the administration of blank phosphoric acid buffer solution which was as self-control.

Table 9: Recovery rate of timolol maleate from GBR solution

Group	No.	Amount added ($\mu g \cdot ml^{-1}$)	Amount observed ($\mu g \cdot ml^{-1}$)	Recovery (%)	Average recovery (%)	RSD (%)
	1	85.23	84.49	99.1		
High	2	85.23	86.56	101.6		
	3	85.23	84.75	99.4		
	4	16.18	16.15	100.0		
Middle	5	16.18	16.07	99.3	100.1	1.70
	6	16.18	15.88	98.1		
	7	0.63	0.62	98.4		
Low	8	0.63	0.64	101.6		
	9	0.63	0.65	103.2		

Table 10: Intra-day precision and inter-day reproducibility of timolol maleate (n = 5)

Concentration ($\mu g \cdot ml^{-1}$)	Intra-day		Inter-day	
	Peak area	RSD(%)	Peak area	RSD(%)
0.64	18072.4	0.8	17675.0	2.7
16	422583.4	0.2	419785.0	0.7
80	2070727.0	0.0	2089530.0	1.3

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References

Chen Y, Wang N (2004) Effect of borneol on the intercellular tight junction and pinocytosis vesicles *in vitro* blood-brain barrier mode. Zhongguo Zhong Xi Yi Jie He Za Zhi 24: 632–634

Di Colo G, Burgalassi S, Zambito Y (2004) Effects of different *N*-trimethyl chitosans on *in vitro/in vivo* ofloxacin transcorneal permeation. J Pharm Sci 93: 2851–2862.

Dong X et al. (2002) Study on the auxo-action of borneol assisting the penetration of DDB across BBB. Chin Pharm J 37: 275–277.

Durand-Cavagna G et al. (1989) Corneal endothelial changes with azone, a penetration enhancer. Lens Eye Toxic Res 6: 109–117.

Fan L et al. (1998) An experiment research on osmosis of cytomembrane of corneal epithelium promoted by Borneolum in the rabbits. J Trad Chin Ophthalmol 8: 67–69.

Fan L, Tang Y, Lu L (2003) Study on enhancer effects of borneol on corneal permeation of aesculin. China J Chin Materia Med 28: 81–82.

Furrer P et al. (2002) Ocular tolerance of absorption enhancers in ophthalmic preparations. AAPS Pharm Sci 4: E2.
Gao J, Yang Q, Li Y (2004) Effect of transdermal enhancers on absorptance of Zushima gaoyao. Chin J New Drugs 13: 416–418.
Jarvinen T et al. (1995) Sulfobutyl ether beta-cyclodextrin (SBE-beta-CD) in eyedrops improves the tolerability of a topically applied pilocarpine prodrug in rabbits. J Ocul Pharmacol Ther 11: 95–106.
Kou Z. of Song Dynasty (1990) Extension of the Materia Medica (ben cao yan yi), 1st ed., Beijing, p. 89–89
Lee VH, Robinson JR (1986) Review: topical ocular drug delivery: recent developments and future challenges. J Ocul Pharmacol 2: 67–108.
Liu Q et al. (1994) The influence of Borneol on the passing of gentamycin through blood-brain barrier. J Guangzhou Univ Trad Chin Med 11: 37–40.
Montenegro L, Bucolo C, Puglisi G (2003) Enhancer effects on *in vitro* corneal permeation of timolol and acyclovir. Pharmazie 58: 497–501.
National Pharmacopoeia Committee (2005a) Pharmacopoeia of the People's Republic of China, 2005th ed., Vol. 2, Beijing, p. 688–689.
National Pharmacopoeia Committee (2005b) Pharmacopoeia of the People's Republic of China, 2005th ed., Vol. 2, Beijing, p. 41–41.
Nomura M et al. (1994) Effect of addition of hyaluronic acid to highly concentrated insulin on absorption from the conjunctiva in conscious diabetic dogs. J Pharm Pharmacol 46: 768–770.
O'Neil M et al. (2001) The Merck Index, 13th ed., New York, p. 224–224.
Rojanasakul Y, Robinson JR (1989) Transport mechanisms of the cornea: characteristic of barrier permselectivity. Int J Pharm 55: 237–240.
Romanelli L et al. (1994) Ocular absorption and distribution of bendazac after topical administration to rabbits with different vehicles. Life Sci 54: 877–885.
Saettone M (2002) Progress and problems in ophthalmic drug delivery. Business Briefing Pharmatech, p. 1–6.
Sasaki H et al. (1999) Enhancement of ocular drug penetration. Crit Rev Ther Drug Carrier Syst 16: 85–146.
Suhonen P et al. (1995) Ocular absorption and irritation of pilocarpine prodrug is modified with buffer, polymer, and cyclodextrin in the eyedrop. Pharm Res 12: 529–533.
Tang-Liu DD et al. (1987) Disposition of levobunolol after an ophthalmic dose to rabbits. J Pharm Sci 76: 780–783.
Tang-Liu DD et al. (1994) Effects of four penetration enhancers on corneal permeability of drugs *in vitro*. J Pharm Sci 83: 85–90.
Wang L, Zhang Q (2002) The study of dipivefrin hydrochloride ophthalmic gel-forming solution. Chin J New Drugs 11: 300–303.
Xu B, Wang H, Xu W (2001) Enhancing effect of synthetic borneol on skin permeation of ligustrazine hydrochloride. Chin Trad Patent Med 23: 865–867.