Ocular Pharmacokinetics of Naringenin Eye Drops Following Topical Administration to Rabbits

Junli Lin,¹ Jingjing Sun,² Yandong Wang,³ Yan Ma,¹ Wenpei Chen,¹ Ziyang Zhang,¹
Gang Gui,¹ and Baojin Lin¹

Abstract

Purpose: To investigate the ocular pharmacokinetics of 1% naringenin eye drops following topical administration to rabbits.

Methods: One drop (50 μ L) of 1% naringenin eye drops was instilled into both eyes of each rabbit. The animals were sacrificed at predetermined intervals after dosing, and ocular tissues and plasma were then collected. Concentrations of naringenin were analyzed using specific electrospray ionization liquid chromatography-tandem mass spectrometry method, which is proved to be sensitive, specific, precise, and suitable for determination of naringenin in ocular tissues and plasma of rabbits.

Results: Ocular exposure to naringenin, based on $AUC_{(0-t)}$, was highest in cornea, followed by aqueous humor, retina, and vitreous body. The C_{max} of naringenin in cornea, aqueous humor, vitreous body, and retina were 67945.30 ± 4109.34 ng/g, 1325.69 ± 239.34 , 160.52 ± 38.78 ng/mL, and 1927.08 ± 660.77 ng/g at 0.083, 0.75, 0.083, and 0.083 h after topical administration, respectively. The half-lives for these tissues were 9.37, 0.65, 1.17, and 4.62 h, respectively. There was no significant difference between free naringenin and total naringenin in plasma based on C_{max} and T_{max} . C_{max} of total naringenin in plasma at 0.083 h was 35.12 ± 0.54 ng/mL.

Conclusions: Measurable concentrations of naringenin were achieved in ocular tissues after topical application in rabbits. Topical instillation of naringenin may be an effective approach in the treatment of posterior section diseases.

Introduction

NARINGENIN, 4',5,7-TRIHYDROXY FLAVANONE (Fig. 1), is richly found in citrus and grape fruits. It has many pharmacological activities such as expectorant activity,¹ antiinflammatory activity,² and DNA protective effect.³ After oral administration of naringenin, naringenin glucuronide was the main existent form in rat plasma. There existed double peaks phenomenon in plasma due to enterohepatic circulation.⁴

Recently, there has been increasing attention on ophthalmological activities of naringenin after topical administration. Topical administration of naringenin markedly reversed $NaIO_3$ -induced retinal pigment epithelium degeneration and laser-induced choroidal neovascularization,⁵ which may attribute to strong increase of ocular blood flow⁶⁻⁸ and antioxidant activity.⁹ Furthermore, our previous study showed that both 0.5% and 1% naringenin eye drops prevented retinal

neurons from *N*-methyl-*N*-nitrosourea (MNU)-induced structural and functional damages.¹⁰ Therefore, naringenin may be a promising molecule for diseases caused by photoreceptor cell death such as age-related macular degeneration (AMD)⁵ and retinitis pigmentosa (RP).¹⁰ To our knowledge, few data about ocular pharmacokinetics of naringenin have been published.

Methods

Materials

Naringenin [purity >98% by high-performance liquid chromatography (HPLC)] and β -glucuronidase ($\geq 1,000,000$ unit/g) were purchased from Sigma-Aldrich Co. Ltd. (St Louis, MO). Hesperidin [purity >98% by HPLC; internal standard (IS)] was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol (HPLC grade) was purchased

¹School of Chinese Materia Medica, Guangzhou University of Chinese Medicine, Guangzhou, China.

²Thomas J. Long School of Pharmacy and Health Sciences, University of the Pacific, Stockton, California.

³Zhongshan Ophthalmic Center, Sun Yat-Sen University, Guangzhou, China.

FIG. 1. Chemical structure of naringenin (4',5,7-trihydroxyflavanone).

from Burdick & Jackson (Honeywell, Muskegon, MI). Other reagents and solvents were analytical grade and HPLC grade, respectively.

Preparation of 1% naringenin eye drops

For the preparation of 1% naringenin eye drops,¹⁰ 10 g of polycarbophil was first added to 400 mL water for injection, stirred, and then swelled for 2 h. The pH of polycarbophil solution was adjusted to 6.0–6.5 with 2 M NaOH and then autoclaved (solution I). Second, 150 g of hydroxypropyl- β -cyclodextrin (HP- β -CD) was dissolved by stirring in 400 mL of water for injection, and then 10 g of naringenin was added and dissolved by sonication for 2 h. Two grams of polox-

amer 407 was added and dissolved by sonication for 0.5 h. Subsequently, 1 g of disodium edentate, 2 g of sodium chloride, and 0.1 g of benzalkonium chloride were added and dissolved (solution II). Finally, solution I and solution II were evenly mixed with stirring and diluted to 1,000 mL with water for injection. After the pH of the mixture was adjusted to 6.50 with 2 M sterile NaOH, the mixture was separated to fill into 100 bottles (Table 1).

Animals

Fifty-two New Zealand white male and female rabbits weighing 2.0–2.5 kg, and free of any signs of ocular inflammation or gross abnormalities, were obtained from Experimental Animal Center, Guangzhou University of Chinese Medicine. The animals had free access to a standard diet and drinking water and were housed in a room maintained at $24.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ and with a 12:12 h cyclic lighting schedule. The experimentation was granted by the Animal Ethics Committee of Guangzhou University of Chinese Medicine, and conformed to the ARVO Resolution for the use of animals in ophthalmic and vision research.

Administration and sample collection

All rabbits were randomly divided into 13 groups according to the following time points: 0, 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 8, 16, and 24 h, 4 rabbits per time point. Four rabbits of 0 h served as control animals and were instilled with normal saline, while other groups were instilled with 1 drop (50 μL) of 1% naringenin eye drops into each

FIG. 2. Product ion spectrum of naringenin (A) and internal standard (B).

TABLE 1. COMPOSITION OF 1% NARINGENIN EYE DROPS

Ingredients	Percentage of composition (w/v) (%)
Naringenin	1.0
HP- β -CD	15
Polycarbophil	1
Poloxamer 407	0.2
Disodium edentate	1
Benzalkonium chloride	0.01
Sodium chloride	0.2

HP- β -CD, hydroxypropyl- β -cyclodextrin.

eye of each rabbit. Blood samples were withdrawn from marginal ear vein after instillation of drug at the corresponding time point. After blood was collected, rabbits were sacrificed immediately and eyes were enucleated and rinsed in normal saline. Aqueous humor was collected and cornea was then harvested at the limbus, rinsed in normal saline, gently wiped, and weighed. One milliliter of vitreous body was obtained, and retina was collected and weighed. Blood samples were anticoagulated by 3.2% sodium citrate solution and then centrifuged at 5,000 rpm for 5 min to obtain plasma. All samples were stored in Eppendorf tubes at -80°C .

Equipment and LC/MS/MS conditions

The liquid chromatography-tandem mass spectrometry (LC/MS/MS) system consisted of an Agilent 1260 liquid chromatograph and a 6460 triple quadrupole mass spectrometer with an electrospray ionization source. Data acquisition was performed with Mass Hunter B 05 software. Chromatographic separation was achieved at 30°C on BDS Hypersil C18 column (100×2.1 mm i.d., $2.2 \mu\text{m}$; Dionex Corporation, Sunnyvale, CA) with a phenomenex C18 guard column (4×2 mm i.d.). The mobile phase consisted of 0.038% ammonium acetate in water (A) and methanol (B) at a ratio of 40:60 (v:v) with a flow rate of 0.2 mL/min. The running time was 4.0 min and the initial 1.5 min was switched to the waste. The injection volume was $5 \mu\text{L}$ and the column was fully eluted after a daily analysis cycle (around 100 samples).

As shown in Figure 2, the mass spectrometer was operated in negative ion mode using multiple reaction monitoring. Transitions (precursor to product) monitored were m/z 271.0/151.0 for naringenin and m/z 608.9/301.1 for IS, respectively. High purity nitrogen was served as both nebulizing and drying gas. Fragmentor and collision energy were 117 V, 14 eV for naringenin and 195 V, 22 eV for IS, respectively. Other mass-spectrometer working parameters were set as follows: drying gas flow, 10 L/min; drying gas temperature, 350°C ; nebulizer pressure, 40 psi; capillary voltage, 3,500 V.

Preparation of calibration standards and quality control samples

Naringenin was precisely weighted and dissolved in methanol to prepare stock solution with a concentration of 1 mg/mL. The stock solution was diluted with methanol to make working solutions over range of 5–2,000 ng/mL. A $10 \mu\text{g/mL}$ solution of IS was also prepared with methanol and then diluted to obtain working solution of 125 ng/mL. All solutions were stored at -20°C .

The calibration standards were prepared by adding appropriate amounts of working solutions (5% of total sample volume) to blank ocular tissues and plasma, and were vortex-mixed for 30 s. The calibration standards were then processed as described in the Sample Extraction section below. At last the calibration standards were made at the concentrations of 1, 2, 4, 8, 25, 50, 100, 200, and 400 ng/mL. The quality control (QC) samples used in the validation had been prepared at the concentrations of 2 ng/mL (low QC), 25 ng/mL (medium QC), and 320 ng/mL (high QC) in the same way that calibration standards were prepared.

Sample extraction

A $100 \mu\text{L}$ aliquot of each aqueous humor, vitreous body, and plasma sample was transferred to an Eppendorf tube with a fixed amount of IS ($10 \mu\text{L}$). Then, $300 \mu\text{L}$ methanol was added to the tube and vortexed for 30 s. After centrifugation at 14,000 rpm for 10 min, the supernatant was transferred to a fresh tube and centrifuged at 14,000 rpm for 10 min again. Next, the supernatant was evaporated to

TABLE 2. CONCENTRATIONS OF NARINGENIN IN OCULAR TISSUES AND PLASMA AFTER TOPICAL ADMINISTRATION 1% NARINGENIN EYE DROPS (MEAN $\pm$ SEM)

Time (h)	Cornea ($\mu\text{g/g}$)	Aqueous humor ($\mu\text{g/mL}$)	Vitreous body ($\mu\text{g/mL}$)	Retina ($\mu\text{g/g}$)	Plasma (ng/mL)	Plasma (enzymolysis) (ng/mL)
0.083	67.9 ± 4.11	0.104 ± 0.0209	0.161 ± 0.0387	1.93 ± 0.661	37.2 ± 5.95	35.1 ± 0.543
0.25	34.7 ± 5.06	0.495 ± 0.139	0.0266 ± 0.00881	0.229 ± 0.0508	23.7 ± 1.64	30.6 ± 2.96
0.5	28.9 ± 1.92	0.905 ± 0.119	0.0194 ± 0.00631	0.217 ± 0.0467	13.8 ± 2.36	15.4 ± 2.58
0.75	31.9 ± 3.85	1.33 ± 0.239	0.0279 ± 0.00648	0.398 ± 0.0565	10.5 ± 2.36	12.5 ± 1.98
1	22.1 ± 2.26	0.565 ± 0.0774	0.0108 ± 0.00349	0.159 ± 0.272	2.89 ± 0.84	11.5 ± 3.31
1.5	17.3 ± 1.62	0.555 ± 0.106	0.0237 ± 0.00739	0.119 ± 0.0164	2.55 ± 0.873	6.66 ± 1.98
2	13.8 ± 2.77	0.288 ± 0.0941	0.00746 ± 0.00130	0.0779 ± 0.0147	1.68 ± 0.425	3.26 ± 0.846
3	7.92 ± 0.463	0.948 ± 0.0173	0.0157 ± 0.00481	0.0545 ± 0.0197	1.39 ± 0.583	1.03 ± 0.146
4	7.18 ± 0.513	0.0343 ± 0.00762	0.0154 ± 0.00473	0.0488 ± 0.0142	1.13 ± 0.678	3.92 ± 1.35
8	5.38 ± 0.759	0.00178 ± 0.000445	0.000575 ± 0.000144	0.0261 ± 0.00809	2.80 ± 0	2.80 ± 0.686
16	1.25 ± 0.187	ND	ND	ND	ND	ND
24	0.963 ± 0.185	ND	ND	ND	ND	ND

ND, not determined, for concentrations of naringenin in rabbit aqueous humor, vitreous body, retina and plasma at 16 and 24 h time point were below LLOQ of the assays (data not shown). $n=8$ for cornea, aqueous humor, vitreous body and retina, and $n=4$ for plasma.

LLOQ, lower limit of quantification; SEM, standard error of measurement.

TABLE 3. PHARMACOKINETIC PARAMETERS OF 1% NARINGENIN EYE DROPS FOLLOWING TOPICAL APPLICATION IN RABBITS

	$C_{\max}/(\text{ng/mL or ng/g})$ (mean $\pm$ SEM)	$T_{\max}/\text{h}$	$T_{1/2}/\text{h}$	K_e/h^{-1}	$AUC_{0-t}/(\text{ng}\cdot\text{h/mL})$ or $\text{ng}/(\text{h}\cdot\text{g})$	MRT_{0-t}/h
Cornea	67945.30 $\pm$ 4109.34	0.083	9.37	0.129	145129.00	7.57
Aqueous humor	1325.69 $\pm$ 239.34	0.75	0.65	0.827	1563.85	1.35
Vitreous body	160.52 $\pm$ 38.78	0.083	1.17	0.517	114.28	2.22
Retina	1927.08 $\pm$ 660.77	0.083	4.62	0.182	848.84	1.73
Plasma	37.25 $\pm$ 5.95	0.083	3.48	-0.109	29.12	2.44
Plasma (enzymolysis)	35.12 $\pm$ 0.54	0.083	8.47	-0.092	46.14	2.65

$C_{\max}$, maximum concentration; $T_{\max}$, time of maximum concentration; $T_{1/2}$, elimination half-life; K_e , elimination rate constant values; AUC_{0-t} , area under the concentration-time curve between 0 and 8 h for aqueous humor, vitreous body, retina and plasma, and between 0 and 24 h for cornea; MRT_{0-t} , mean residence time between 0 and 8 h for aqueous humor, vitreous body, retina and plasma, and between 0 and 24 h for cornea.

dryness under a stream of nitrogen at 40°C, and the final residue was dissolved in 100 μL methanol with vortex-mixing for 30 s. Each mixture was centrifuged 14,000 rpm for 10 min and 5 μL supernatant was injected to the LC/MS/MS via the autosampler for analysis. The concentrations of some samples above the linearity were diluted with methanol into the range of working calibration curve before injected to the LC/MS/MS.

Cornea and retina samples were thawed and homogenized with methanol (1 g:10 mL) by IKA T25, Digital Ultra-Turrax (Staufen, Germany) at 80,000 rpm for 30 s. Then, 100 μL aliquot of each homogenized tissue sample was mixed with 10 μL IS. The following preparation steps were the same as sample extraction from aqueous humor, vitreous body, and plasma.

Total naringenin (including naringenin and its glucuronides) in plasma was obtained by incubating 100 μL plasma with β -glucuronidase (10 μL) at 37°C for 1 h. The following extraction steps were the same as sample extraction from aqueous humor, vitreous body, and plasma.

Analytical method validation

Analytical method validation was performed on rabbit cornea, aqueous humor, vitreous body, retina, and plasma. The method was validated for specificity and selectivity, linearity, precision, accuracy, extraction recovery, and stability according to the U.S. Food and Drug Administration guidelines for the validation of bioanalytical methods.^{11–13}

Pharmacokinetic analysis

The pharmacokinetic parameters, such as the maximum concentration ($C_{\max}$) and the time of maximum concentration ($T_{\max}$) were directly obtained from concentration-time plots. The elimination half-life ($T_{1/2}$), elimination rate constant (K_e), area under the curve from the time of dosing to the last measurable concentration (AUC_{0-t}), and the mean residence time (MRT) were calculated by using noncompartmental pharmacokinetic analysis of mean concentration values of all samples from each group with DAS (Drug and statistics for windows, Version 2.0, Chinese Pharmacological Association)

FIG. 3. Concentration-time curves of naringenin in rabbit (A) cornea, (B) aqueous humor, vitreous body, and retina, and (C) plasma after topical administration of 1% naringenin eye drops. Values were shown as mean $\pm$ standard error of measurement; $n=8$ for cornea, aqueous humor, vitreous body, and retina; $n=4$ for plasma.

program. Data were expressed as mean $\pm$ standard error of measurement and values below lower limit of quantification (LLOQ) were excluded during data analysis.

Results

Method validation

In this study, after conducting protein precipitation, we found that the extraction method provided good recoveries for naringenin from rabbit ocular tissues and plasma without detectable interference. The LC/MS/MS qualifying naringenin in rabbit ocular tissues and plasma was validated, which was sensitive, specific, precise, and suitable. Consequently, above methods could be applied to study pharmacokinetics after topical instillation of 1% naringenin eye drops.

Pharmacokinetic analysis

The concentrations of naringenin in rabbit ocular tissues and plasma had been summarized in Table 2 and pharmacokinetic parameters were summarized in Table 3. The data in Figure 3 clearly showed naringenin mean concentration versus time profile in rabbit ocular tissues and plasma after topical administration of 1% naringenin eye drops. However, concentrations of naringenin in rabbit aqueous humor, vitreous body, retina, and plasma at 16 and 24 h time points were below LLOQ of the assays (data not shown).

Discussion

Topical administration of naringenin markedly reversed NaIO₃-induced retinal pigment epithelium degeneration and laser-induced choroidal neovascularization.⁵ Our previous study has shown that 0.5% or 1% naringenin eye drops prevented retinal neurons from MNU-induced structural and functional damages.¹⁰ To our knowledge, few data about ocular pharmacokinetics of naringenin have been published. In this study, the ocular pharmacokinetic profile of 1% naringenin eye drops after its topical administration in rabbits was characterized.

HP- β -CD, one of the most useful additives in ophthalmic formulations, increases aqueous solubility and stability of some lipophilic drugs without changing their molecular structure.¹⁴ Naringenin is sparingly soluble in aqueous buffers. Therefore, by using HP- β -CD, naringenin is soluble in the preparation of 1% naringenin eye drops. Polycarbophil is an amphipathic polymer of polyacrylic acid, which has been extensively described for its bioadhesiveness¹⁵ and increasing retention of formulation in the eye for some drugs.^{16,17} In this study, MRT of naringenin in cornea was 7.57 h, longer than other tested ocular tissues (Table 3), which may be due to mucoadhesive polycarbophil formulation.

Since it is hard for most drugs to reach therapeutic levels in intraocular tissues due to many ocular barriers, blood flow and efflux transporter and others eye drops are slowly developed in the treatment of posterior section diseases such as AMD and RP.^{18,19} *In vitro* study clearly showed that 1 μ g/mL naringenin increased the proliferation of retinal pigment epithelium cells and inhibited the growth of human umbilical vein endothelial cells.⁹ In this study, $C_{\max}$ of naringenin in retina was 1927.08 $\pm$ 660.77 ng/g at 0.083 h (the collection time of first sample), suggesting that naringenin can reach retina with measurable and therapeutic concentration after topical instillation.

There are 2 routes of drug delivery to the posterior segment for topical ophthalmic drugs. Transcorneal route (cornea $\rightarrow$ aqueous humor $\rightarrow$ intraocular tissues $\rightarrow$ choroid/retina) is generally considered to be primary for amphipathic or small molecules. Conjunctival-scleral route (conjunctiva $\rightarrow$ sclera $\rightarrow$ ciliary body $\rightarrow$ choroid/retina) is another route and important in the absorption of some hydrophilic or large compounds.^{20–24} Concentration of naringenin in retina (1927.08 $\pm$ 660.77 ng/g) was higher than that in aqueous humor (103.89 $\pm$ 20.92 ng/mL) at 0.083 h ($P < 0.05$). $T_{\max}$ and $C_{\max}$ of aqueous humor were 0.75 h and 1325.69 $\pm$ 239.34 ng/mL, respectively. These results suggest that naringenin penetrates both cornea and conjunctiva/sclera into the posterior segment. The reason is most likely that naringenin is a small molecule lipophilic drug. Furthermore, HP- β -CD, acting as a penetration enhancer by promoting drug availability at the surface of biological barrier,^{25,26} helps naringenin penetrating through cornea and conjunctiva/sclera.

Following topical administration of eye drops, systematic pharmacokinetics should be determined to indicate the systematic adverse reaction. Previous study showed that naringenin glucuronides was the main existent form in rat plasma and glucuronidation occurs during the first pass after oral administration.⁴ Therefore, the free and total naringenin were assessed in rabbit plasma after topical application of 1% naringenin eye drops in our study. However, in this study, there was no significant difference between free naringenin and total naringenin in plasma based on $C_{\max}$ and $T_{\max}$ (Table 3), which may be because the absorption route of naringenin bypasses the liver after topical instillation to eyes. Moreover, $C_{\max}$ of total naringenin and AUC_{0–8h} in plasma were 35.12 $\pm$ 0.54 ng/mL and 46.14 ng/(h $\cdot$ mL) by topical instillation in rabbits, respectively; while $C_{\max}$ of total naringenin and AUC_{0–48h} in plasma were 16977.78 ng/mL and 30990.94 ng/(h $\cdot$ mL) by oral administration at low dosage of 30 mg/kg in rats, respectively.⁴ These results suggest that incidence of systematic adverse reaction of naringenin is extremely low.

Conclusions

A highly sensitive and rapid LC/MS/MS method for quantitation of naringenin in rabbit ocular tissues and plasma was developed and validated. Measurable and therapeutic levels of naringenin can be achieved in rabbit retina after topical administration, indicating that naringenin eye drops can be a promising drug for the treatment of posterior section diseases.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Dr. Baoqin Lin

School of Chinese Materia Medica

Guangzhou University of Chinese Medicine

Guangzhou 510006

China

E-mail: linbqcpu@126.com; linbaoqin@gzhtcm.edu.cn