Trans-Scleral Permeability of Oregon Green 488®

Sung Jin Lee,^{1,2} Stephen J. Kim,² Esther S. Kim,² Dayle H. Geroski,²
Bernard E. McCarey,² and Henry F. Edelhauser²

Abstract

Purpose: The aim of this study was to determine the scleral permeability of a commercially available version of 2',7'-difluorofluorescein (OG) and compare it to that of sodium fluorescein (NaF).

Methods: Both *in vitro* and *in vivo* experiments were performed. For the *ex vivo* experiment, a Lucite block perfusion chamber with human donor sclera was used. Two hundred microliters (200 μ L) of 2.5 mg/ml OG or NaF was placed in the donor chamber. The OG and NaF concentration that diffused across the sclera was measured every 2 h for 24 h by fluorometry, and the fluorescence in the sclera was examined by fluorescent microscopy. *In vivo* experiments consisted of live rabbits treated with a 0.2-mL subtenon injection of 7.5 mg/ml solution of either OG or NaF in the right eye. Intraocular fluorescence was measured by ocular fluorophotometry.

Results: The scleral permeability coefficient (K_{trans}) of OG was $3.93 \pm 1.01 \times 10^{-7}$ cm/sec and that of NaF was $4.41 \pm 1.32 \times 10^{-7}$ cm/s. Both OG and NaF were visible throughout the sclera after 24 hours. Peak vitreous concentration after subtenon injection in rabbits was 6.48 ± 2.65 ng/mL of OG at 2 min and 47.15 ± 13.3 ng/mL of NaF at 10 min.

Conclusions: OG was able to diffuse across the sclera and thus could be potentially useful as a fluorescent tag for intraocular drug delivery studies. However, its permeability was substantially less than that of NaF.

Introduction

SODIUM FLUORESCIN (NaF) has been an invaluable diagnostic tool for retinochoroidal disease. More recently, it has been used to investigate the trans-scleral delivery of drugs administered periocularly. By labeling drugs with NaF, accurate detection and measurement of drug concentration under both *in vitro* and *in vivo* conditions can be achieved. Specifically, the permeation of labeled drug through donor sclera can be measured accurately via fluorometry, labeled drug concentration in the vitreous can be measured via ocular fluorophotometry, and the presence of drug within the sclera can be detected with fluorescein microscopy.^{1,2} Even though NaF has proven useful in drug-delivery research, it is limited by photobleaching³ and pH sensitivity,⁴ and thus, more stable, alternative fluorescent agents would be beneficial.

Oregon Green 488® (OG; 2',7'-difluorofluorescein, Molecular Probes, Eugene, OR) is a newly developed fluorescent

agent that overcomes the photobleaching and pH sensitivity of NaF (AK-Fluor®; Buffalo Grove, IL) and has the additional advantage of higher fluorescence.⁵ Further, since OG has nearly the same structure, molecular weight, and emission and excitation characteristics as NaF (Table 1), efficient utilization of existing equipment and research protocols should be feasible.

OG has already been extensively conjugated to many substrates, including chemicals, antibiotics, proteins, and nucleic acids, and employed successfully in various research applications instead of NaF. However, OG has had limited application in ophthalmic research, to date, and has never, to our knowledge, been studied in ocular drug delivery.^{6–10}

Therefore, to investigate its potential application in drug-delivery research, we first evaluated the trans-scleral permeability and pharmacokinetics of OG, compared with NaF, using both *ex vivo* and *in vivo* experimental models.

¹Department of Ophthalmology, College of Medicine, Soonchunhyang University, Seoul, Korea.

²Department of Ophthalmology, Emory University, School of Medicine, Atlanta, GA.

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TABLE 1. CHARACTERISTICS OF SODIUM FLUORESCEIN (NaF) AND OREGON GREEN 488® (OG)

	NaF	OG
Structure
Formula	C ₂₀ H ₁₂ Na ₂ O ₅	C ₂₀ H ₁₀ F ₂ O ₅
MW (g/mol)	376.27	368.29
Excitation max (nm)	495	493
Emission max (nm)	517	520

MW, molecular weight.

Methods

Preparation of OG NaF

The molecular weight of OG is 368.29 and that of NaF is 376.28. For both *in vitro* and *in vivo* experiments, 2.5 mg of either OG or NaF was dissolved in 1 mL of balanced salt solution (BSS; Alcon Laboratories, Fort Worth, TX) under sterile conditions.

In vitro scleral permeability

Twenty (20) human donor scleral specimens (Georgia Eye Bank, Atlanta, GA) were excised and stored in moist chambers for no longer than 2 days. Five (5) scleral specimens were tested with OG and NaF each, and another 10 were tested with BSS to serve as controls. Scleral preparation and *in vitro* diffusion setup were performed, as previously described.² In brief, episclera and uveal tissue were carefully removed. The sclera was mounted in a specially designed Lucite block perfusion chamber. The perfusion apparatus clamped the sclera between a donor chamber on the episcleral side and a receiver chamber on the choroidal side. The choroidal side was perfused continuously at a rate of 0.03 mL/min with BSS. Two hundred microliters (200 μ L) of OG, NaF, or BSS was applied to the episcleral surface. The perfusate was collected in a fraction collector every 2 h and analyzed by a fluorometer to determine the fluorescein concentration that had diffused through the sclera.

The diffusion constant, K_{trans} (measured in centimeters per second), was measured at the points of stable flux for OG and NaF in each delivery vehicle and was calculated as follows:

$$K_{trans} = [R_{total}/(A \times t)] \times [1/D]$$

where R_{total} equals the total moles through sclera in time t (s); A the surface area of the sclera (cm^2); and D the concentration of original solution in the donor chamber (moles/mL).

In vitro scleral absorption

To investigate drug absorption, the sclera was removed from the perfusion chamber after *in vitro* permeability testing and bisected. One of the bisected sclera halves was embedded in low-viscosity epoxy medium and then frozen in dry ice. Unstained frozen sections were then prepared and

examined with a fluorescent microscope (Nikon, Melville, NY). Photomicrographs were taken (magnification, $\times 400$) to demonstrate the spatial distribution of drug absorbed by the sclera. These were compared with photomicrographs of control sclera exposed to BSS.

In vivo subtenon injection

This experiment was performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Visual Research. Ten (10) Dutch-belted rabbits, 2.2 kg in weight, were used in all the experiments. The rabbits were anesthetized with 6 mg/kg of xylazine and 15 mg/kg of ketamine intramuscularly before subtenon injection.

Subtenon injection of 0.2 mL of OG or NaF (7.5 mg/mL) was administered in the right eye of rabbits. A total of 5 right eyes received OG and another 5 right eyes received NaF. Subtenon injection was performed in the superotemporal quadrant by using a tuberculin syringe with a 30-gauge needle. The needle was introduced along the sclera beyond the equator, and the material was then slowly injected. In each case, there was a localized ballooning of the subtenon space 5–6 mm away from the limbus with no significant reflux of material. The injection site was compressed by using a cotton swab after the injection. The left eye of each animal served as a control and was not injected.

Copious irrigation of the cornea and conjunctiva with at least 500 mL of BSS was performed after the injection. An external ocular exam, slit-lamp exam, and dilated fundus exam was performed before and immediately after the injection to evaluate for any inflammation or drug reaction.

Baseline fluorescein concentration in the anterior segment, vitreous, and the choroid/retina was measured in all rabbits in both eyes, using fluorophotometry (Fluorotron Master fluorophotometer; OcuMetrics, Mountain View, CA) with a standard objective lens. Fluorescein concentration in various ocular tissues (e.g., retina/choroid, vitreous, and cornea) was obtained at specific intervals that were 0.25 mm apart (1 interval) along an optical axis by the fluorophotometer. The fluorophotometer has an internal standard and gives absolute fluorescein concentrations at each interval. It provides linear fluorescence concentration values between 0 and 2000 ng/mL. For the purposes of this study, the midvitreous fluorescence level was defined as the maximum fluorescence between 16 and 20 intervals (4–6 mm) anterior to the retina/choroid peak and the anterior-segment fluorescence level was defined as the maximum fluorescence between 16 and 20 (4–6 mm) intervals posterior to the corneal peak. The term anterior segment was used instead of lens, iris, or aqueous humor in this study, because it is hard to precisely confirm where the fluorescence is with regard to tissue site.

Intraocular fluorescein concentration was measured at the following time points after injection: 10 and 30 min and 1, 2, 3, 5, 7, 24, and 48 h until fluorescence returned to baseline levels. All measurements were performed under nonanesthetized conditions, and 3 scans were taken at each time point.

Statistical analyses

All average values are recorded as the mean $\pm$ standard error of the mean. The Student *t*-test was used to determine the significance between mean values. $P < 0.05$ was considered significant.

FIG. 1. Concentration of fluorescein (mean $\pm$ standard error of the mean; $n = 5$) in the receptor tube after trans-scleral penetration of sodium fluorescein (A, NaF) and Oregon Green 488® (B, OG) with relationship to time. OG diffuses into the sclera slower and less than NaF. The scleral permeability coefficient (K_{trans}) of NaF was $4.41 \pm 1.32 \times 10^{-7}$ and that of OG was $3.93 \pm 1.01 \times 10^{-7}$ cm/s.

Results

In vitro scleral permeability

Figure 1 shows the concentration (ng/mL) of OG or NaF detected in the perfusate sampled at 2-h intervals from the receiving chamber. The diffusion of NaF across the sclera at 2 h already demonstrates a difference than the BSS con-

trol group, reaching a peak concentration at 4 h (12.67 ± 6.4 ng/mL) and achieving steady state (8.0–9.0 ng/mL) between 14 and 20 h (Fig. 1A). In contrast, the permeation of OG was similar to the control for the first 4 h, reached its peak concentration later at 8 h (9.43 ± 3.6 ng/mL), and achieved steady state (7.3–7.7 ng/mL) between 18 and 22 h (Fig. 1B). The level of fluorescence of the respective con-

FIG. 2. Scleral absorption of Oregon Green 488® (OG) and sodium fluorescein (NaF). Fluorescein microscopic findings with light exposure at 50 ms show strong fluorescence in whole human sclera with NaF (A) and OG (C) and faint autofluorescence in sclera with balanced salt solution control (B, D). OG-exposed sclera shows stronger fluorescence than NaF.

TABLE 2. THE OREGON GREEN 488® (OG) AND SODIUM FLUORESCIEIN (NaF) CONCENTRATION IN OCULAR TISSUES AFTER 0.2-mL SUBTENON INJECTION OF (5 MG/mL)

	Baseline	10 min	30 min	1 h	2 h	3 h	5 h	7 h	1 day	2 days	
OG (OD)	Retina/choroid	5.04 ± 1.90	5.68 ± 1.24	5.72 ± 1.67	12.29 ± 3.45	12.34 ± 3.77	9.15 ± 1.31	7.99 ± 1.13	5.89 ± 1.12	3.45 ± 0.78	2.99 ± 0.56
	Vitreous	0.84 ± 0.20	1.49 ± 0.39	1.38 ± 0.60	2.31 ± 1.0	6.48 ± 2.65	4.52 ± 1.72	2.09 ± 1.45	1.09 ± 0.27	0.56 ± 0.30	0.61 ± 0.30
	Anterior segment	0.52 ± 0.55	2.27 ± 0.66	2.43 ± 0.92	2.52 ± 0.76	2.48 ± 0.56	2.55 ± 0.38	1.98 ± 0.37	1.65 ± 0.24	1.71 ± 0.18	1.13 ± 0.35
Control	Retina/choroid	5.41 ± 2.90	5.46 ± 1.97	5.20 ± 1.65	5.53 ± 1.56	4.56 ± 1.19	4.69 ± 1.86	4.82 ± 2.40	4.16 ± 0.77	3.17 ± 0.94	3.21 ± 1.16
of OG	Vitreous	0.83 ± 0.34	0.94 ± 0.44	1.11 ± 0.38	0.96 ± 0.25	1.25 ± 0.25	1.38 ± 0.28	1.11 ± 0.16	0.93 ± 0.27	0.61 ± 0.35	0.67 ± 0.37
(OS)	Anterior segment	1.87 ± 0.63	2.11 ± 0.98	1.36 ± 0.56	1.33 ± 0.40	1.28 ± 0.38	1.40 ± 0.55	1.38 ± 0.60	1.11 ± 0.47	1.19 ± 0.43	1.10 ± 0.34
NaF (OD)	Retina/choroid	6.30 ± 1.41	264.41 ± 123.32	211.93 ± 61.65	159.44 ± 79.43	119.63 ± 53.88	58.82 ± 39.40	18.66 ± 5.74	10.23 ± 1.78	1.31 ± 1.70	5.50 ± 0.52
	Vitreous	1.89 ± 0.26	47.15 ± 13.30	37.22 ± 20.97	23.26 ± 9.43	10.48 ± 1.35	9.52 ± 1.12	4.61 ± 1.99	1.88 ± 0.34	0.51 ± 0.13	0.69 ± 0.18
	Anterior segment	2.14 ± 0.61	5.43 ± 0.64	3.28 ± 0.86	3.99 ± 1.18	3.78 ± 0.73	3.03 ± 0.95	2.55 ± 0.48	2.54 ± 0.46	1.43 ± 0.62	2.09 ± 0.60
Control	Retina/choroid	5.58 ± 1.51	5.54 ± 4.95	5.31 ± 4.39	6.00 ± 2.32	4.98 ± 2.01	4.33 ± 1.50	4.87 ± 1.90	1.90 ± 0.37	0.44 ± 0.29	4.71 ± 0.11
of NaF	Vitreous	0.88 ± 0.32	1.47 ± 0.33	4.06 ± 0.89	7.53 ± 2.32	1.02 ± 0.21	1.06 ± 0.04	1.08 ± 0.57	1.39 ± 0.19	0.50 ± 0.20	0.39 ± 0.13
(OS)	Anterior segment	2.08 ± 0.96	2.77 ± 0.43	2.31 ± 0.38	2.51 ± 0.62	2.74 ± 1.25	2.39 ± 0.92	2.19 ± 1.07	2.02 ± 0.77	2.12 ± 0.39	1.39 ± 0.13

Data are the mean concentration of fluorescein (ng/mL) ± the standard deviation. The mean peak values are shown in bold (n for OG = 5; n for NaF = 4).

trol groups (exposed to BSS) for both NaF and OG were similar.

From these data, the steady-state permeability constant for trans-scleral diffusion (K_{trans}) was calculated as $4.41 \pm 1.32 \times 10^{-7}$ and $3.93 \pm 1.01 \times 10^{-7}$ cm/s for NaF and OG, respectively.

In vitro scleral absorption

Scleral fluorescein microscopic photographs after 50 m of light exposure show strong fluorescence throughout the sclera with NaF (Fig. 2A) and OG (Fig. 2C), but only trace autofluorescence in BSS treated sclera (Fig. 2B and 2D). OG-exposed sclera shows stronger fluorescence than NaF exposed sclera.

In vivo subtenon injections

The maximum fluorescence values (ng/mL) of the anterior segment, midvitreous, and retina/choroid for OG- and

NaF-treated eyes were analyzed and are summarized (mean $\pm$ standard deviation) in Table 2.

Figure 3 diagrams the mean fluorescence levels in intraocular tissues after subtenon injection of 0.2 mL of 7.5 mg/mL of NaF and OG. The baseline fluorescence in the retina/choroid before injection was 5–6 ng/mL, whereas the baseline fluorescence in the vitreous and the anterior segment was 1–2 ng/mL. The maximum fluorescein concentration in the retina/choroid reached 264.41 ± 123.32 ng/mL at 10 min after subtenon injection of NaF and 12.29 ± 3.45 ng/mL at 1 h after injection of OG. The vitreous level peaked at 47.15 ± 13.30 ng/mL 10 min after NaF injection and 4.68 ± 0.61 ng/mL 2 h after OG injection. The anterior segment concentration peaked at 5.43 ± 0.64 ng/mL 10 min after NaF injection, and peaked at 2.55 ± 0.38 ng/mL 3 h after OG injection.

Figure 4 compares the peak NaF and OG concentrations of the retina/choroid, midvitreous, and anterior segment versus time with that of the contralateral control eye. The

FIG. 3. Fluorophotometric scan of intraocular sodium fluorescein (NaF) (A) and Oregon Green 488® (OG) (B). Retina/choroid concentration of NaF peaked at 10 min (264.41 ± 123.32 ng/mL) and OG at 2 h (12.34 ± 3.77 ng/mL). Midvitreous concentration of NaF peaked at 10 min (47.15 ± 13.30 ng/mL) and OG at 2 h (4.68 ± 0.61 ng/mL) after subtenon injection. The fluorescence returned to the baseline level at 7 h.

FIG. 4. Fluorophotometry scans of Oregon Green 488® (OG) and sodium fluorescein (NaF) concentrations in retina (A, B), midvitreous (C, D), and anterior segment (E, F) versus time. The graphs suggest that the vitreous and anterior-segment concentrations were directly influenced by the retinal concentration. The NaF concentration in each case achieved rapid peak levels but returned rapidly to baseline, while OG concentrations demonstrated a slower peak and decline.

retina/choroid concentration of OG reached its peak at 2 h after injection, maintained peak levels for about 2 h, and decreased to baseline after 7 h, compared with control eyes. The retina/choroid concentration of NaF, on the other hand, demonstrated peak concentration at 10 min after injection, with a more rapid decrease and return to baseline after 7 h. The vitreous concentration of OG after subtenon injection reached its peak at 2 h and returned to its baseline after 5 h, whereas NaF peaked at 10 min and returned to baseline af-

ter 7 h. The anterior-segment concentration of OG maintained peak levels 4 h after injection and returned to the baseline by 5 h, and NaF reached peak levels at 10 min and thereafter was not statistically different from control eyes.

Discussion

Trans-scleral delivery of drugs reaches the choroid from the subtenon space by direct diffusion across the sclera^{11,12}

and crosses the blood-retinal barrier into the vitreous. This delivery is affected by the drug's molecular weight, radius, partition coefficient, and charge. From previous research, the subtenon route achieves the highest vitreous concentrations among periorbital injections with the lowest systemic drug levels¹ and thus offers the potential for efficient, safe drug delivery to treat posterior segment disease.

Investigating the pharmacokinetics of trans-scleral drug delivery has been aided tremendously by fluorescein labeling. Fluorescein has been used to conjugate many ocular drugs, such as antibiotics, dexamethasone, methotrexate, dextran, oligonucleotide, and ovalbumin, in order to investigate the trans-scleral drug pharmacokinetics for posterior segment disorders.^{13–15} However, fluorescein is easily photobleachable³ and pH-sensitive,⁴ which can make it difficult to combine with some drugs and unstable.

OG is a fluorinated analog of fluorescein. Two hydrogens are substituted for two fluorines at the 2' and 7' positions of fluorescein (2',7'-difluorofluorescein). This change makes OG less pH sensitive and much more photostable than fluorescein. The molecular weight of NaF (376.27 g/mol) is similar to OG (368.29 g/mol) and the absorption and emission spectra of both are nearly identical, which makes changing the parameters or filters of the fluorometer and fluorophotometer used for NaF unnecessary and allows for easy adaptation of OG. Thus, OG may offer a convenient, useful alternative to NaF in trans-scleral drug-delivery studies.

Consequently, we directly compared the *in vitro* and *in vivo* trans-scleral pharmacokinetics of OG with NaF. The fact that previous research by Gbate and colleagues¹ reported peak vitreous concentration of NaF at 3.5 h (in stark contrast to 10 min in this study) after posterior subtenon injection emphasizes the importance of proper internal controls, which were performed in this study.

We found key differences in the pharmacokinetics of trans-scleral delivery between NaF and OG. NaF demonstrates greater scleral permeability than OG. The retina/choroid and vitreous concentration of NaF was 22- and 7-fold higher than OG, respectively. This dramatic difference in permeability may be explained by the chemical structure of OG (two fluorines instead of two hydrogens and two sodiums). Moreover, NaF achieved rapid and higher peak levels in the retina/choroid than OG and demonstrated more rapid clearance. In contrast, OG demonstrated a more delayed peak in the retina/choroid, reached a significantly lower peak concentration than NaF, and showed slower clearance. Further, this difference in pharmacokinetics between NaF and OG in the retina/choroid was directly paralleled in the vitreous and anterior segment. Specifically, the vitreous concentration of OG peaked 1 h after the retina/choroid peak (2 h after injection), and the anterior segment concentration peaked 2 h after the vitreous peak (at 4 h); but after NaF injection, the retina/choroid, vitreous, and anterior segment all peaked within 30 min, indicating rapid, efficient diffusion of NaF through the sclera, blood-retinal barrier, and intraocular tissue.

Although the rabbit model is commonly used for *in vitro* ocular drug-delivery studies, it is important to highlight its limitations. Compared with human eyes, the rabbit eye has thinner scleral thickness (2/3 thickness), smaller vitreous volume, and higher choroidal flow rates.^{1,17,18} However, the scleral permeability, and the anatomy and physiology of the

lymphatic system, is similar.^{19,20} Ultimately, how these differences would effect the pharmacokinetics of trans-sclerally delivered drugs in human eyes is difficult to predict, but nevertheless, the rabbit model is an established, useful model for estimating trans-scleral drug pharmacokinetics.

Conclusions

In conclusion, Oregon Green 488 penetrates the sclera, crosses the blood-retinal barrier after subtenon injection, and showed no evidence of toxicity. The vitreous and anterior-segment concentration of OG was directly influenced by the retina/choroid concentration. The results of this study demonstrate the pharmacokinetic differences between OG and NaF after subtenon injection and should serve as an important reference point for the future interpretation of trans-scleral drug-delivery studies utilizing OG.

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Reprint Requests: Henry F. Edelhauser
 Department of Ophthalmology
 Emory University
 School of Medicine
 1365 B Clifton Road NE, Suite B3410
 Atlanta, GA 30322

E-mail: Ophthfe@emory.edu