Effect of Benzalkonium Chloride on Transscleral Drug Delivery

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PURPOSE. To investigate the effect and safety of benzalkonium chloride on transscleral drug delivery in the rabbit after continuous intrascleral administration.

METHODS. Betamethasone 21-phosphate (BP) aqueous solutions, with or without benzalkonium chloride (BAK), were continuously administered to albino rabbit sclera with an osmotic pump for 1 week. The BP concentrations in the aqueous humor, vitreous, and retina-choroid were measured by high-performance liquid chromatography (HPLC). To investigate the effect of BAK on scleral permeability of BP in vitro, penetration of BP aqueous solution with or without BAK across the rabbit sclera was evaluated using a two-chamber Ussing apparatus. To determine the effects of BAK on transscleral delivery of large molecules, 20- and 70-kDa fluorescein isothiocyanate (FITC)-dextran (FD-20 and -70, respectively) aqueous solutions, with or without BAK, were continuously administered to the sclera by an osmotic pump. The intensity of fluorescence in the aqueous humor, vitreous, and retina-choroid was measured by fluorescence spectrophotometry at 1 week after implantation of the pump. The retinal toxicity of BAK was evaluated electrophysiologically and histologically.

RESULTS. BAK increased concentrations of BP in the vitreous and retina-choroid compared with the control. BP was not detected in the aqueous humor. In the in vitro study, BAK did not increase the scleral permeability of BP. In the retina-choroid, BAK significantly increased concentrations of FD-20 but did not increase those of FD-70. The addition of BAK did not increase concentrations of FD-20 or -70 in the vitreous. No substantial toxic reactions were observed in the retina in electrophysiological or histologic examinations after the addition of BAK.

CONCLUSIONS. The results of this study demonstrate that BAK may improve the ocular penetration of a drug in a transscleral drug delivery system without producing toxic reactions. (Invest Ophthalmol Vis Sci. 2005;46:703–708) DOI:10.1167/iovs.03-0934

Several intraocular diseases, such as uveitis, diabetic retinopathy, and proliferative vitreoretinopathy, necessitate long-term treatment with a drug at the site of the disordered intraocular tissues. However, physiological barriers in the eye make it difficult to keep effective concentrations of a drug in the eye for an extended time by conventional methods such as instillation, topical injection, and systemic administration. For long-term drug therapy, intravitreal drug-delivery systems such as microspheres and implants have been investigated.1–4 These devices, however, carry potential side effects of retinal detachment, endophthalmitis, and cataract due to their administration as well as their injection.

Recently, it has been hypothesized that transscleral drug delivery may be an effective method to achieve therapeutic concentrations of drugs in the posterior part of the eye.3–7 We have recently demonstrated that steroid-loaded biodegradable intrascleral implants can deliver a drug into the vitreous and retina-choroid in therapeutic concentrations.8 These findings have suggested that the transscleral route is useful for intraocular drug delivery without severe adverse effects. However, it is speculated that transscleral drug penetration may be affected by physical and chemical characteristics of the drugs, such as molecular weight, water solubility, and lipophilicity.9,10 Some drugs may be inappropriate for the transscleral delivery system, because they must penetrate through the sclera, choroids, and retina.6–7,9–11

Preservatives and surfactants such as benzalkonium chloride (BAK), polysorbate 80, and EDTA have been instilled in droplets for ocular diseases over long periods and have been considered relatively safe. These additives also have played a role as absorption enhancers to improve drug penetration through the cell membrane by acting primarily on the tight junctions.12–14 In this study, we investigated the effects of BAK (cationic surfactant) on the transscleral delivery of BP by using an osmotic pump in the rabbit. In general, it is believed that surfactants affect membrane fluidity and enlarge the intracellular spaces.12–14

We determined the effects of BAK on the scleral permeability of BP in vitro using a two-chamber Ussing apparatus. Furthermore, we investigated in the rabbit the effects of BAK on transscleral delivery by osmotic pump of the high-molecular-mass compounds 20- and 70-kDa fluorescein isothiocyanate (FITC)-dextran (FD-20 and -70, respectively). The retinal toxicity of BAK was evaluated by electrophysiological and histologic examinations.

MATERIALS AND METHODS

Betamethasone 21-phosphate (BP), BAK, and FITC-dextran (molecular mass, 20 and 70 kDa) were purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals were of reagent grade.

Osmotic Pump Implantation

All animals were handled according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Albino rabbits were anesthetized with a mixture (1:1) of xylazine hydrochloride (2 mg/kg) and ketamine hydrochloride (5 mg/kg). The ocular surface was then
the eyes were enucleated. The eyes were immediately frozen at
intravenous pentobarbital sodium at 1 week after implantation, and the
without 0.01% or 0.05% BAK. Animals were killed with an overdose of
pump containing BP in phosphate-buffered saline (50 mg/mL), with or
Twenty-five eyes of 30 albino rabbits, weighing 2.0 to 2.5 kg each,
Concentrations of BP in Ocular Tissue
Twelve rabbits receiving 0.05% or 0.5% BAK, with (n = 3) or without
into the HPLC column, as described. Under these conditions, the
detection limits for BP and BM were 100 ng/g in the aqueous humor
and retina-choroid and 10 ng/g in the vitreous. The BP concentrations
In Vivo Ocular Permeability of Large Molecules
Twenty-four eyes of 24 albino rabbits, weighing 2.0 to 2.5 kg each,
Concentrations of BP and betamethasone (BM), a metabolite of BP,
were determined in recovered ocular tissues (aqueous humor, vitreous,
retina-choroid) by the described HPLC procedures. BP and BM
were extracted from the tissues by the following process: One-tenth
milliliter of internal standard solution (2.5 μg/mL) and 3.0 mL of 0.2 M
HCl were added to each tissue sample. The mixture was homogenized
and centrifuged at 3000 rpm for 15 minutes (KN-70; Kubota, Tokyo,
Japan). The supernatant was collected, and BM was extracted twice
with 3.0 mL of ethyl acetate. Ethyl acetate phases were then dried
under reduced pressure with a centrifugal concentrator (VC-960;
Taitec Co., Saitama, Japan). The residue was dissolved with 0.2 mL
mobile phase. One hundred microliters of this solution was injected
into the HPLC column, as described. Under these conditions, the
detection limits for BP and BM were 100 ng/g in the aqueous humor
and retina-choroid and 10 ng/g in the vitreous. The BP concentrations
in ocular tissues were represented as the total BP and BM molecules per gram of wet tissue.

In Vitro Scleral Permeability Study
Albino rabbits were killed with an overdose of intravenous sodium
centorbarbital, and the eyes were enucleated. The sclera was dissected and mounted in a two-chamber Ussing apparatus (Vidrex Co., Ltd.,
Fukuoka, Japan). Physiologic saline (BSS Plus; Alcon Laboratories, Fort
Worth, TX) containing 5 mM BP, with or without an absorption enhancer (0.01% or 0.05% BAK) was added to the orbital side. The
saline was added to the uveal side. The contents of each chamber were
stirred gently. The Ussing chamber apparatus was incubated at 37°C. Samples (200 μL) were withdrawn from the uveal side at 15-minute intervals for 4 hours, and the drug concentration was measured by
HPLC, as described.

Determination of the Permeability Coefficient
Diffusion from the orbital side to the uveal side through the sclera was characterized by means of a permeability coefficient (P₀), which is the ratio of steady state flux to the concentration gradient. The BP concentration in the uveal-side chamber (Cᵤ) was <1% of its concentration in the orbital-side chamber (Cₒ). Thus, the change in Cₒ was assumed to be the limit of detection, and the permeability coefficient was therefore calculated as follows: $P₀ = \frac{Cₒ - Cₒ₃}{(V/Cₒ₅)}$. The rate of appearance of BP on the vitreous side was calculated by subtracting the initial concentration of the system at equilibrium ($Cₒ₃$) from the final concentration measurement at 4 hours ($Cₒ₃$). V is the volume of each chamber (5 mL), t is the duration time of steady state flux converted from hour to second, and S is the surface area of exposed sclera (0.6 cm²).

In Vivo Ocular Permeability of Large Molecules
Twenty-four eyes of 24 albino rabbits, weighing 2.0 to 2.5 kg each,
were used. Only the right eye of each rabbit received the osmotic pump containing FD-20 or -70 in phosphate-buffered saline (250 mg/
ml) with or without 0.01% BAK. Animals were killed with an overdose of intravenous pentobarbital sodium at 1 week after implantation, and the eyes were enucleated. The eyes were immediately frozen at ~85°C, and samples of ocular tissues (aqueous humor, vitreous, and retina-
choroid) were retrieved. The ocular tissues were stored at ~85°C until the fluorescence intensity was measured. Fluorescence in all the ocular tissues was measured at room temperature (25°C) with a fluorescence spectrophotometer (model FP-6200; Jasco Corp., Tokyo, Japan). Excitation and emission wavelengths were 495 and 525 nm, respectively. Corrections were made for tissue autofluorescence by using fluorescence levels in the normal eye.

Light Microscopy
Twelve rabbits receiving 0.05% or 0.5% BAK, with (n = 5) or without
(n = 3) 50 mg/mL BP aqueous solution, using an osmotic pump were killed at 1 week after implantation. The eyes were enucleated and immediately immersed in a mixture of 4% glutaraldehyde and 2.5% neutral-buffered formalin for 24 hours. Globes were opened at the pars plana, and the cornea, lens, and vitreous were carefully removed. The recovered retina-choroid and sclera beneath the administration site were dehydrated, infiltrated, embedded in paraffin, and sectioned with a microtome. Sections were stained with hematoxylin-eosin for light microscopy.

Electron Microscopy
Four rabbits receiving 0.05% BAK, with (n = 2) or without (n = 2) 50 mg/mL BP aqueous solution, by osmotic pump were killed at 1 week

Figure 1. Sketch of a rabbit eye showing the projected implantation site of the osmotic pump.
after implantation. With the rabbits under deep anesthesia, the eyes were enucleated and immediately immersed in 2.5% glutaraldehyde-loaded 0.1 M phosphate buffer. Globes were opened at the pars plana, and the cornea, lens, and vitreous were carefully removed. The retina-choroid beneath the administration site was dissected and cut into small pieces ~1 to 2 mm², and immersed in the same fixative for 2 hours. The samples were washed twice with 0.1 M phosphate buffer (pH 7.4) before being postfixed in 2% osmium tetroxide and 0.1 M phosphate buffer for 2 hours at 4°C. After dehydration in a graded series of ethanol, the samples were embedded in Epon 812 (TAAB Laboratories; Aldermastron, UK) and subsequently cut with a diamond knife on an ultramicrotome (Ultracut-E; Reichert-Jung, Heidelberg, Germany), double stained with uranyl acetate and lead citrate, and examined under an electron microscope (JEM-1200 EX; JEOL Co., Tokyo, Japan).

Electrophysiological Study
Retinal function was evaluated by scotopic electroretinography (ERG) before and 1 week after administration by osmotic pump of 0.05% and 0.5% BAK containing BP aqueous solution. Scotopic ERG was performed after 60 minutes of dark adaptation (ERG-50; Kowa Co., Ltd, Nagoya, Japan). A silver-plated electrode was placed on each earlobe, forming after 60 minutes of dark adaptation (ERG-50; Kowa Co., Ltd, Nagoya, Japan). A silver-plated electrode was placed on each earlobe, with one serving as the reference and the other as the ground. The ERG responses were analyzed by dividing the b-wave amplitudes recorded from the contralateral, control eyes.

Statistical Analysis
An unpaired t-test was used to assess whether concentrations of BP, FD-20, and -70 had increased in the ocular tissues with addition of BAK. An unpaired t-test was also used to assess whether the permeability coefficient of BP in the rabbit sclera had increased with the addition of the absorption enhancer in vitro. A paired t-test was used to assess whether the ratio of the b-wave amplitude of the treated eye in comparison with the control eye had decreased after treatment. \( P < 0.05 \) was considered statistically significant.

RESULTS
Effect of Absorption Enhancers on Ocular Permeability of BP
The concentrations of BP in the vitreous and retina-choroid increased with the addition of 0.01% and 0.05% BAK (Table 1). BP was not detectable in the aqueous humor throughout the study (detection limit: 100 ng/g). BAK increased concentrations of BP in the vitreous and retina-choroid in a dose-dependent manner (Table 1).

<table>
<thead>
<tr>
<th>Absorption Enhancers</th>
<th>Vitreous BP Concentration (µg/g)</th>
<th>Retina-Choroid BP Concentration (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additives</td>
<td>0.02 ± 0.01</td>
<td>1.18 ± 0.56</td>
</tr>
<tr>
<td>0.01% BAK</td>
<td>0.13 ± 0.13*</td>
<td>4.95 ± 1.36*</td>
</tr>
<tr>
<td>0.05% BAK</td>
<td>0.43 ± 0.35*</td>
<td>12.54 ± 5.96*</td>
</tr>
</tbody>
</table>

- Data were recorded after continuous intrascleral administration of BP, with and without 0.01% or 0.05% benzalkonium chloride (BAK), using osmotic pumps. The data are mean ± SD of \( n = 4–6 \). BP, betamethasone 21-phosphate.
- \( * P < 0.05 \), \( ** P < 0.01 \).

In Vitro Scleral Permeability Study
Scleral permeability of BP was assessed at 15-minute intervals for 240 minutes using the Ussing chamber. The permeability coefficient \((\times 10^{-6} \text{ cm/sec})\) was 5.30 ± 0.94 (mean ± SD, \( n = 4 \)) and 4.78 ± 0.81 for BP, with or without 0.05% BAK, respectively (Fig. 2). BAK did not significantly increase the permeability of sclera \((P = 0.20)\).

In Vivo Ocular Permeability of Large Molecules
Addition of 0.01% BAK significantly increased concentrations of FD-20 in the retina-choroid but not in the vitreous (Table 2). Concentrations of FD-70 in the vitreous and retina-choroid did not increase significantly with the addition of 0.01% BAK (Table 2). Neither FD-20 nor FD-70 was detected in the aqueous humor.

Toxicity Studies
Figure 3 shows the light micrograph of the retina around the administration site of 0.05% and 0.5% BAK aqueous solution. The 0.05% BAK aqueous solution revealed no substantial changes in the retina, with (data not shown) or without BP. However, retinal detachment was observed after the administration of 0.5% BAK solution, with (data not shown) or without BP. Figure 4 showed transmission electron micrographs of the retina around the administration site of 0.05% BAK aqueous solution. The retina maintained a normal structure. The tight junction between the retinal pigment epithelial cells and the microvilli of retinal pigment epithelial cells demonstrated no significant structural changes.

Right-to-left (experimental eye/control eye) ratios of the scotopic b-wave before administration were 1.16 ± 0.14 and 0.94 ± 0.13 (mean ± SD) in the eyes receiving BP aqueous solution containing 0.05% and 0.5% BAK, respectively. The right-to-left ratios of the scotopic b-wave after administration were 1.11 ± 0.35 and 0.76 ± 0.12 in the eyes receiving BP aqueous solution containing 0.05% and 0.5% BAK, respectively. Although no significant difference was observed between ratios before and after administration of BP aqueous solution containing 0.05% and 0.5% BAK, the right-to-left ratios of the scotopic b-wave after administration in the eyes receiving BP aqueous solution containing 0.5% BAK decreased.

![Figure 2. The effect of BAK on scleral permeability of BP in vitro, determined with a two-chamber Ussing apparatus. Mean ± SD; \( n = 4 \).](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932931/ on 12/02/2018)
**TABLE 2. Concentrations of Fluorescein Isothiocyanate-Dextran 20-kDa (FD-20) or 70-kDa (FD-70) in the Vitreous and Retina-Choroid**

<table>
<thead>
<tr>
<th>Absorption Enhancer</th>
<th>FD-20 Concentration (nmol/g)</th>
<th>FD-70 Concentration (nmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vitreous</td>
<td>Retina-Choroid</td>
</tr>
<tr>
<td>No additives</td>
<td>0.011 ± 0.009</td>
<td>1.133 ± 1.029</td>
</tr>
<tr>
<td>0.01% BAK</td>
<td>0.014 ± 0.007</td>
<td>2.455 ± 1.057*</td>
</tr>
</tbody>
</table>

Data were collected after continuous intrascleral administration of FD-20 or FD-70, with and without 0.01% benzalkonium chloride (BAK), using osmotic pumps. The data are means ± SD of n = 4–6. *P < 0.05.

**DISCUSSION**

In this study, we demonstrated the effects of BAK as one of the absorption enhancers on the scleral permeability of BP in the rabbit, by using an osmotic pump. In our preliminary study, BAK showed the most significant effect on the intraocular penetration of BP by the transscleral delivery in all studied absorption enhancers, which were BAK, EDTA, and polysorbate 80 (data not shown). The addition of BAK increased concentrations of BP in the vitreous and retina-choroid in a dose-dependent manner. Concentrations of large molecules (20 kDa) in the retina-choroid were also enhanced by the addition of BAK. Our results suggested that transscleral drug delivery may be facilitated by the addition of BAK.

To determine the mechanism of increased ocular permeability with absorption enhancers, we investigated the effect of BAK on the scleral permeability of BP in vitro using an Ussing chamber. The permeability coefficient of the sclera was not significantly improved by the addition of 0.01% or 0.05% BAK. BAK did not increase the scleral permeability of BP. In vivo, BAK significantly increased BP concentrations in the vitreous and retina-choroid. The enhancement ratios in the vitreous were higher than those in the retina-choroid. Hydrophilic low molecular compounds such as BP may diffuse easily from the retina to the vitreous. It is likely that BAK may increase the penetration of BP from the choroid to the retina. In this study, however, we did not evaluate the effect of absorption enhancers on the tight junction of the retinal pigment epithelium (RPE), because we did not measure concentrations in the retina and choroid separately. It is believed that cationic surfactants, such as BAK, improve drug penetration of the cell membrane by acting primarily on the tight junctions. Therefore, BAK may also act on the tight junctions in the RPE and increase BP concentrations in the vitreous and retina-choroid.

Recently, transscleral delivery of macromolecules has been studied. Ambati et al. reported that scleral permeability decreased with increasing molecular weight and molecular radius in vitro. They also reported that large molecules, such as IgG, could be delivered across the sclera and show biological effects in vivo. Aihara et al., Kim et al., and Weinreb demonstrated that the scleral permeability of a large-molecule compound increased with prostaglandin (PG) or PG analogue exposure. In this study, we investigated the effect of BAK on the transscleral delivery of large-molecule FD-20 and -70. The addition of 0.01% BAK significantly increased concentrations of FD-20 in the retina-choroid compared with the control. However, concentrations of FD-70 in the retina-choroid did not increase significantly with the addition of 0.01% BAK. These findings suggest that the addition of 0.01% BAK may not improve ocular permeability of macromolecules larger than 70 kDa. Furthermore, the addition of BAK did not increase concentrations of FD-20 and -70 in the vitreous. BAK may not enhance the permeability of the internal limiting membrane, because the internal limiting membrane and the sclera are mainly composed of collagen fiber.

We evaluated the toxic effects of BAK on the retina histologically and electrophysiologically. In general, the effect of most absorption enhancers has been associated with histologic damage to the biological membrane. Chou et al. have reported that BAK reduces the a- and b-wave amplitudes in electroretinograms in pigmented rabbits and induces retinal detachment, visual cell loss, and atrophy of the RPE and choroid. Miyake et al. have reported that BAK may be responsible for pseudophakic cystoid macular edema due to disruption of the blood–aqueous barrier. Recently, Pisella et al. reported that the inflammatory marker in the conjunctival cell of patients with glaucoma after instillation of eyedrops containing 0.02% BAK for at least a year was significantly increased compared with that after instillation of eyedrops without BAK. However, BAK is most frequently used in commercial eyedrops because of its rapid bactericidal efficacy and low toxicity under properly controlled conditions. In addition, BAK has often been investigated as an absorption enhancer in the cornea and conjunctiva. Sasaki et al. reported that the addition of 0.01% and 0.05% BAK increased the permeability of thyrotropin-releasing hormone (TRH) and luteinizing hormone-releasing hormone (LHRH) through the cornea and the conjunctiva in rabbits. In the present study, although retinal toxic reactions were observed after administration of 0.5% BAK aqueous solution, no substantial changes were observed histologically and electrophysiologically after administration of 0.05% BAK aqueous solution. In the conjunctiva after admin-
istration of 0.05% BAK, no abnormal changes, such as edema or congestion, were observed (data not shown). In this study, we delivered the 0.05% BAK aqueous solution into the sclera with an osmotic pump, which released the solution at a constant rate (0.25 \( \mu L/h \)). The total volume released from the osmotic pump in a week is almost equivalent to 1 drop (40 \( \mu L \)) from the ophthalmic droplets. Therefore, the use of 0.01% or 0.05% BAK may be tolerable in a sustained drug-delivery system through the sclera. However, further safety studies may be necessary before clinical use.

In this study, we used an osmotic pump to evaluate the effect of absorption enhancers on transscleral drug delivery, because this device releases the drug at a constant rate. Clinically, however, more feasible transscleral drug-delivery systems are required. We have recently developed two types of transscleral drug-delivery devices, intrascleral implants and posterior episcleral implants. Betamethasone, with a molecular mass of 355 Da, could be delivered into the retina-choroid system through the sclera. However, further safety studies may be necessary before clinical use.

References


