JOURNAL OF OCULAR PHARMACOLOGY AND THERAPEUTICS
Volume 22, Number 5, 2006

The Effect of Recombinant Human Hyaluronidase on Dexamethasone Penetration into the Posterior Segment of the Eye After Sub-Tenon's Injection

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ABSTRACT

***Purpose*: The aim of this study was to investigate the extent if recombinant human hyaluronidase (rhuPH20) can enhance trans-scleral penetration of sub-Tenon's dexamethasone (DM) into the posterior segment of the eye.**

***Methods*: rhuPH20 was purified from conditioned media through a series of ion exchange, hydrophobic interaction, aminophenylboronate, and hydroxyapatite chromatography to greater than 90% purity based upon specific activity. Only the right eye of each rabbit was injected. The first group (n = 16) received an injection of DM and rhuPH20, whereas the second group (n = 16) received DM only. The eyes were enucleated 1, 2, 3, and 6 h after the injection, and the choroid, retina, vitreous, aqueous, and serum were harvested. DM concentration was assessed by mass spectrometry. Histology (n = 2) and immunohistochemistry (n = 2) was performed to detect toxicity and the presence of the rHuPH20, respectively.**

***Results*: We observed no histopathologic damage to ocular tissues after sub-Tenon's injection. This enzyme significantly increased DM level in the choroid and the retina 3 h after administration. The rise in levels was transient returning to normal levels by 6 h.**

***Conclusions*: Sub-Tenon's coinjection of rHuPH20 with DM resulted in a general increase in DM levels in ocular tissues and the serum, with significant increase in the choroid and the retina, 3 h after administration.**

INTRODUCTION

NEW METHODS FOR DRUG DELIVERY to the posterior segment of the eye have generated specific interest in the ophthalmic community.[1] Developing more effective ocular drug delivery methods with less threatening complications is essential to improving the treatment of posterior segment eye disease. A challenge of drug delivery is targeting specific tissues without systemic spread of the compound, which many times, in doses capable of achieving therapeutic levels in the eye, may cause unacceptable side-effects. Topical eye drop treatment of posterior segment eye diseases is often ineffective owing to low ocular tissue permeabilities, drug dilution in tears, unwanted counterdirectional convection of drugs, and long diffusion path lengths.[2,3] Intravitreal drug delivery, although effective, is an invasive technique with risks for ocular infections and tissue damage.[4,5] In addition, only a limited volume of drug can be delivered this way.

Trans-scleral corticosteroid drug delivery has been successfully used for treatment of posterior

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Halozyme Therapeutics, Inc., San Diego, CA, provided unrestricted support for this research.

segment disease. The routes of administration include subconjunctival,[6] retro- or peribulbar[7] and sub-Tenon's injection.[8] However, for a drug to reach the posterior segment following periocular injection, it must first diffuse across the sclera. The rate and extent of intraocular penetration are primarily dependent on the permeability of the sclera. Based on previous studies, the scleral tissue is permeable to a wide molecular weight range.[9] In addition, it has been shown that therapeutic genes can be delivered by the trans-scleral route using adenoviral vectors.[10] The sclera has a large and accessible surface area, a high degree of hydration rendering it conducive to hydrophilic molecules, is hypocellular with paucity of proteolytic enzymes, and has a permeability that does not appreciably decline with age.[11–15]

Existing methods of trans-scleral delivery are either nontargeted or destructive. Drugs injected into the subconjunctival space reach intraocular tissues[16–18] but typically do so through anterior segment vascular plexes/systemic circulation,[7] and often, do not reach sustained therapeutic levels in the choroid or retina. Trans-scleral iontophoresis can cause retinal necrosis and gliosis in the area of application.[19] It is time-consuming and is safe with only low-current densities.[20] Sub-Tenon's injection a offers reasonable means of capitalizing on trans-scleral drug delivery while minimizing the dangers of systemic administration. Recently, the ability to facilitate trans-scleral drug delivery has been reported in a study by Okabe and colleagues,[21] who showed that the preservative, benzalkonium chloride, improves drug ocular penetration in a trans-scleral drug delivery system without producing toxic reactions.

In this study, we hypothesized that human recombinant hyaluronidase would be a nontoxic facilitator of delivery of therapeutic concentrations of dexamethasone (DM) into the posterior pole choroid, retina, and the vitreous after sub-Tenon's injection. Hyaluronidase, a hydrolytic enzyme, is, in ophthalmology, commonly added to anesthetic agents administered using peri- and retrobulbar techniques to enhance diffusion through the orbital tissues.[22] The disadvantage of the pharmacy-compounded hyaluronidase available to ophthalmologists is its animal origin (until recently, bovine and now ovine), which might be toxic if delivered to the ocular contents.[23–25] The human recombinant form, as developed by Frost and coworkers, is devoid of antigenic properties.[26] We have used this bioengineered form of hyaluronidase in our experiments to determine how well it permeabilizes the sclera to allow for the penetration of dexamethasone.

METHODS

Preparation of human recombinant hyaluronidase and DM

A cDNA clone encoding a soluble, neutral active form of the human PH20 gene (rhuPH20) was identified from the full-length human PH20 cDNA by screening progressive carboxy-terminal truncations of rhuPH20 for soluble neutral active enzyme activity when transfected into Chinese hamster ovary (CHO) cells. Enzyme activity in culture supernatants were measured as described previously.[26] An expression system was constructed as a cytomegalovirus-based bicistronic cassette driving both the soluble rhuPH20 domain (amino acids 1–447) and the murine dihydrofolate reductase (DHFR) gene separated by an internal ribosomal entry site. The selected clone was electroporated into $DHFR^{-/-}$ CHO cells (DG44) previously adapted to grow in a chemically defined, animal product–free medium (VPSFM™, GIBCO/Invitrogen; Carlsbad, CA). Cells were cloned by limiting dilution and amplified in methotrexate. rhPH20 was purified from conditioned media through a series of ion exchange, hydrophobic interaction, amino-phenylboronate, and hydroxyapatite chromatography to greater than 90% purity based upon specific activity ($>$ 90,000 USP units/mg). CHO host protein contaminants were less than 1% by enzyme-linked immunosorbent assay (ELISA; Pharmingen, San Jose, CA), and preparations had an endotoxin content less than 5 EU/mg protein. Enzyme potency in bulk preparations was determined by turbidimetric assay with the United States Pharmacopeia (USP) hyaluronidase HA reference standard. Analysis of HA fragment size following digestion with rhuPH20 was performed, as described previously.[26]

In 3-mL syringes, rhuPH20 (3000 units) was combined with DM (10 mg/mL; American Pharmaceutical Partners, Schaumburg, IL), at a ratio of $<$ 1% (volume dexamethasone:volume human recombinant hyaluronidase). In other syringes, DM was loaded into 3-mL syringes in combination with less than 1% saline.

Animals and injections

A total of 36 eyes of 34 New Zealand rabbits were used in the study. All animal handling was in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The rabbits weighed 2.1–3.2 kg. For surgical procedures and ocular examinations, the rabbits were anesthetized with intramuscular injections of ketamine hydrochloride (21 mg/kg) and xylazine (5.25 mg/kg). Topical 0.5% proparacaine was used for anesthetizing ocular surface. Pupils were dilated with 1% tropicamide and 2.5% phenylephrine.

Four (4) eyes of 2 rabbits were injected with rhuPH20 for histopathologic toxicity study and the same number was used for immunohistochemical analysis. From the pharmacokinetics group, only the right eye of each rabbit were injected. The first group (n = 16) received an injection of DM and PH20, whereas the second group (n = 16) received DM only. Four (4) eyes were injected for every time point. After opening of conjunctiva with Vannas (World Precision Instruments, Sarasota, FL) scissors, the tip of tri-port anesthetic Mendez cannula (Eagle Laboratories, Rancho Cucamonga, CA) was inserted into sub-Tenon's space, and 1.5 mL of drug was injected adjacent to the posterior pole of the eye. A cotton-tipped applicator was placed at the site of conjunctival entry to prevent egress of fluid.

For euthanasia, the rabbits were anesthetized as described above and sacrificed by intracardiac injection of pentobarbital sodium (120 mg/kg) at 1, 2, 3, and 6 h after the injection. Upon euthanasia, 1 mL of blood sample was taken and both eyes were enucleated from each animal. The external sclera was dried using gauze. Immediately after the enucleation, a 2-mL sample of aqueous humor was collected through anterior chamber paracentesis with a tuberculin syringe from each eye. After removal of the anterior segment from enucleated eyes, 1 mL of vitreous was withdrawn and placed into labeled conical polypropylene tubes. The remainder of the eye was dissected and a 6-mm trephine (Fine Science Tools; Foster City, CA) was used to punch the tissue sample inferior to the optic nerve where drug was injected on the extrascleral side. From the punched tissue, the retina was dissected off with a blunt blade and placed into a separate labeled tube and the choroid placed into another separate tube. Instruments were alcohol cleaned and dried after each eye.

Histopathology

After enucleation, the globe was postfixed for 2–10 h in a solution containing 2% paraformaldehyde/2% glutaraldehyde in phosphate-buffered saline (PBS; pH 7.2) or in 4% paraformaldehyde in PBS (pH 7.0) at 4°C. Retinal toxicity and vitreous inflammation were evaluated from paraffin sections stained with hematoxylin-eosin.

Drug concentration analysis

Sample processing procedure. This analysis was done in a masked fashion so that investigators did not know if the analyzed tissue was with or without rhuPH20. For the choroid and retinal samples, a buffer containing 50 mM Tris base, 1 mM $MgCl_2$, pH 9.0, was added to the tissue, so the final concentration was 20 mg/mL. The tissue was disrupted using an ultrasonic homogenizer (BioLogics, Inc., Manassas, VA, model 150 V/T) for 5–15 sec. The samples of tissue homogenate, the vitreous fluid, aqueous fluid, and plasma were all analyzed by the following procedure. Samples containing DM and prednisolone (PN) as the internal standard (IS) were extracted with methyl *t*-butyl ether by vortex mixing for 5 min. The organic and aqueous layers were separated by centrifugation at 1300*g* for 5 min, the aqueous layer was frozen at −70°C for 15 min, and the organic layer was poured into new tubes and evaporated under a stream of nitrogen at 40°C. The residue was reconstituted with water:acetonitrile (50:50 v/v). The processed

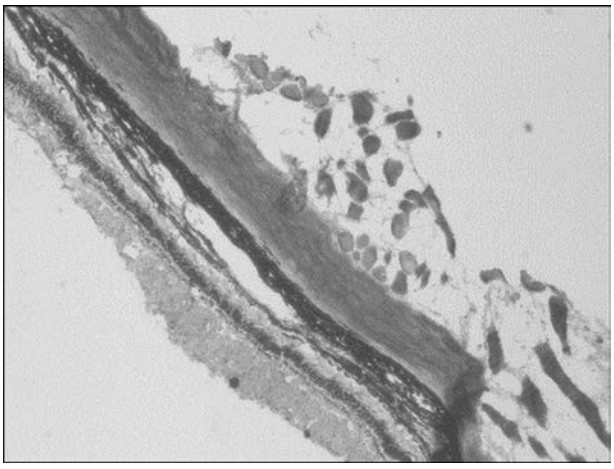

FIG. 1. Microphotograph of the posterior rabbit retina after sub-Tenon's injection of human recombinant hyaluronidase reveals no histopathologic damage to retina tissue; Hematoxyllin and Eosin, 20×.

samples were analyzed by high-performance liquid chromatography using a Phenomenex Synergi Polar RP (Bratislava, Slovakia) column maintained at 45°C. The mobile phase was nebulized using heated nitrogen in a Z-spray source/interface. The ionized compounds were detected in electrospray-positive mode using a tandem quadrupole mass spectrometer with transitions of 393.40 > 237.19 for DM and 361.30 > 147.09 for PN (cone = 40 V and collision energy = 20 eV for both).

Calculations

Peak heights of both compounds were acquired and integrated using MassLynx™ version 4.0 software (Micromass; Beverly, MA). The calibration curves were obtained by fitting the peak height ratios of the standards to the IS a power equation in MassLynx. The equation of the calibration curve was then used to interpolate the concentration of DM in the samples using their peak height ratios.

Statistics

Samples for each time point were averaged and the means were compared using one-tailed *t* test or the Mann-Whitney test, according to the distributions of the data. P-values less than 0.05 were considered statistically significant. Area under the curve (AUC) was calculated using the trapezoidal rule.

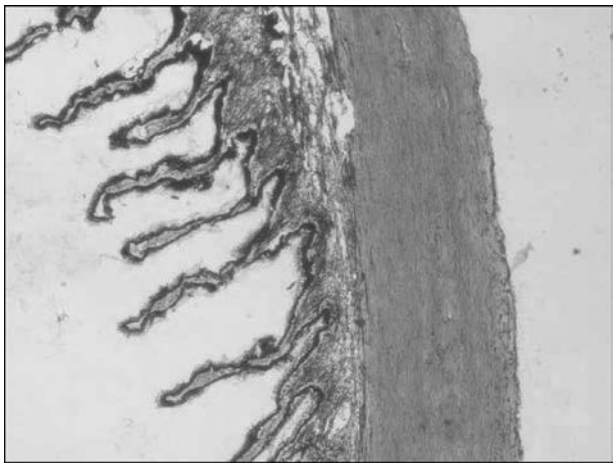

FIG. 2. Microphotograph of the rabbit pars plicata after sub-Tenon's injection of human recombinant hyaluronidase reveals no histopathologic damage to adjacent tissue; Hematoxyllin and Eosin, 20×.

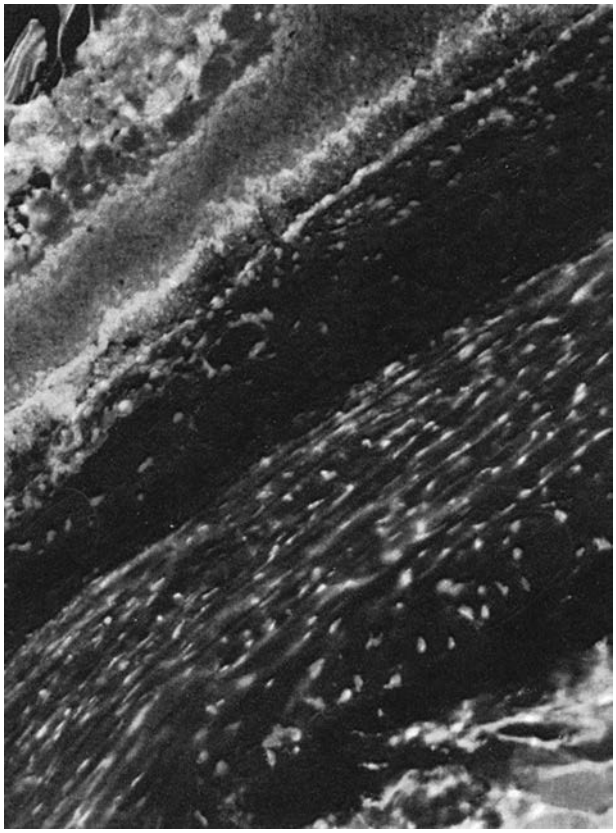

FIG. 3. Immunostained photomicrograph of the eye wall and the posterior segment of the rabbit eye using biotinilated hyaluronidase binding protein counterstained with (4′, 6-diamidino-2-phenylindole (DAPI)/fluorescein isothiocyanate (FITC)-avidin showing plentiful hyaluronan in the sclera and choroid.

RESULTS

All 36 animals survived well during the experiment and no complications of the injections, such as hemorrhage or bulbus penetration, were observed. Figures 1 and 2 show that no histopathologic damage occurs in the posterior retina and the pars plicata structures in rabbits after the injection of rhuPH20. Figures 3 and 4 show distribution of hyaluronidase in the eye wall and posterior segment before and after sub-Tenon's injection. Figures 5-7 show average ± standard deviation of concentrations of DM in the choroid, retina, and the vitreous, as measured by mass spectrometry after predetermined time points. The results indicate that in the majority of time points in all tissues examined (the choroid, retina, vitreous, aqueous, and the serum), the level of DM was higher when coinjected with the rhuPH20. Statistically significant differences in favor of rhuPH20 were

FIG. 4. Immunostained photomicrograph of the eye wall and the posterior segment of the rabbit eye using biotinilated hyaluronidase binding protein counterstained with DAPI/FITC-avidin 3 hours after sub-Tenon's injection of human recombinant hyaluronidase showing dissolved hyaluronan compared to Figure 3.

found in the retinal tissue at 3 h ($P = 0.001$) and in the choroid also at 3 h ($P = 0.03$) and the vitreous after 1 h ($P = 0.05$; Figs. 5, 6). Whereas these data points were significantly higher for the DM + rhuPH20 than DM alone, the AUC analysis did not show an overall increase in drug levels. The AUC with the rhuPH20 for the choroids, retina, and the vitreous was 1729 ng.h/mL, 365 ng.h/mL, and 9390 ng.h/mL, respectively. The AUC without the enzyme for the choroids, retina, and vitreous was 1736 ng.h/mL, 246 ng.h/mL, and 11,493 ng.h/mL, respectively.

DISCUSSION

Corticosteroids are the mainstay of therapy for many diseases of the retina and posterior segment of the eye. Previous studies with extraocular DM administration show that the highest concentration in subretinal fluid occurs 3 h after subconjunctival injection,[6] and after peribulbar injection,[7] the highest levels in the vitreous were achieved after 6 h. We have, therefore, concentrated on pharmacokinetic analysis during the period of the first 6 h after trans-scleral administration, which was confirmed by our unpublished animal data obtained before commencement of this experiment.

Addition of hyaluronidase has been used in anesthesia to improve tissue penetration of anesthetic solutions by depolymerizing the hyaluronic acid present in the intracellular matrix.[15] Hyaluronic acid and hyaluronan have been described in the Bruch's membrane, chorioretinal complex, the sclera, and perimysial connective tissue of extraocular muscle.[27,28] Hyaluronidase used in this study is human based[26] and has been cloned and purified to avoid unwanted adverse reactions of animal-based compounds.[23–25] Indeed, administration of rhuPH20 in this experiment has not been associated with any clinically detectable changes in periocular tissues of animals, and our toxicity studies showed no intraocular changes either.

The sclera containes collagen fibrils and proteoglycans that contain approximately 70% water. Solutes traverse the tissue through the interfibrillar aqueous media of the gel-like proteoglycans. Molecular size is, therefore, an important limiting factor in the diffusion of drugs.[9]

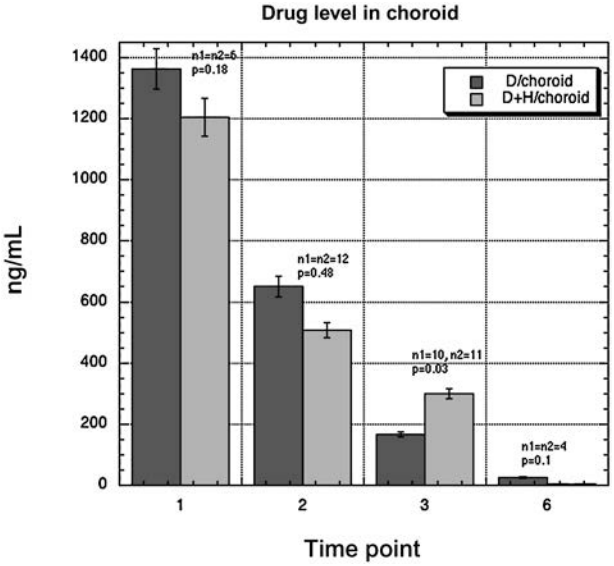

FIG. 5. The comparison of dexamethasone levels in the rabbit choroid at different time points after sub-Tenon's injection of dexamethasone alone versus dexamethasone and rhuPH20.

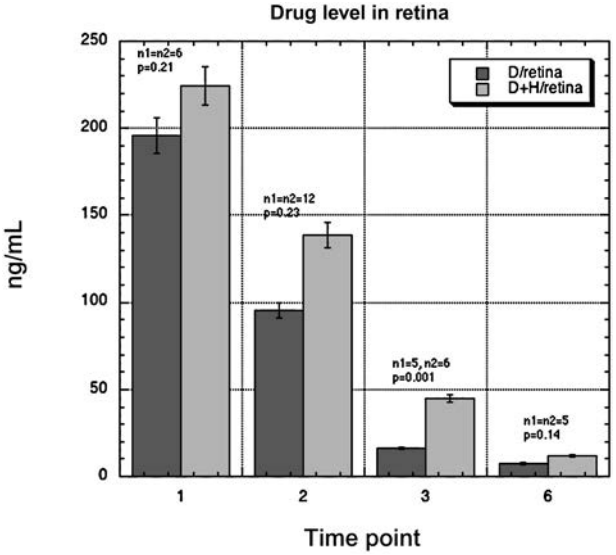

FIG. 6. The comparison of dexamethasone levels in the rabbit retina at different time points after sub-Tenon's injection of dexamethasone alone versus dexamethasone and rhuPH20.

Because of the large surface area and high degree of hydration, the sclera provides an aqueous pathway through which hydrophilic molecules can pass.[29] The permeability of human sclera to DM in balanced salt solution (BSS) plus is $11.5 \pm 1.1 \times 10^{-6}$ cm.sec^{-1} (12) or $23.5 \pm 7.7 \times 10^{-6}$ cm.sec^{-1} in another study.[13] The presumed mechanism of action of human recombinant hyaluronidase in this experiment is that rhuPH20 "opens" interfibrillar spaces through which DM molecules can reach intraocular tissues in greater amount than when administered alone without rhuPH20.

We observed that this begins approximately at 3 h after extraocular administration of the compound (DM + rhuPH20). We found significantly higher DM levels in the retinal tissue and in the choroid. The vitreous also showed significantly higher levels of DM when PH20 was used at 1 h postinjection. The rest of tissues in the majority of time points showed only a trend toward higher penetration of DM when injected with rhuPH20 rather than DM alone (data not shown for the aqueous and the serum). The AUC calculation did not reveal a difference between rhuPH20 facilitated and nonfacilitated DM levels. The increased levels of DM in the choroid and the retina at 3 h after injection did not last until the next time point. Therefore, it may not be enough for a transient increase in the drug's levels to contribute to statistical significance of the whole AUC.

In clinical application, both the retina and the choroid are our target tissues, which need high drug levels. We used DM as a prototype drug for trans-scleral delivery, but other drugs can be used as well. The choroid is known for its high blood flow where retention of any drug may be problematic. This and likely fast diffusion further intraocularly caused why we did not detect DM levels in the choroid comparable to the retina. Another explanation can be that the enzyme helps to diffuse dexamethasone not only in the intraocular direction, but in the opposite direction (i.e., into the orbit as well). DM levels in the orbital tissues were not assayed in this experiment. However, an increased DM level after rhuPH20 was detected in the blood serum, which points to widespread distribution of dexamethasone after coinjection with enzyme.

The vitreous is a structure with relatively stable metabolic turnover. Because it is the last to be reached after trans-scleral administration of any drug in nonvitrectomized eyes, it is there where we would anticipate its lowest levels, compared to the choroid and the retina. Last but not least, even though each step of the experiment strictly followed the described protocol, we cannot exclude the possibility of slight variation in either the injection technique or tissue harvesting.

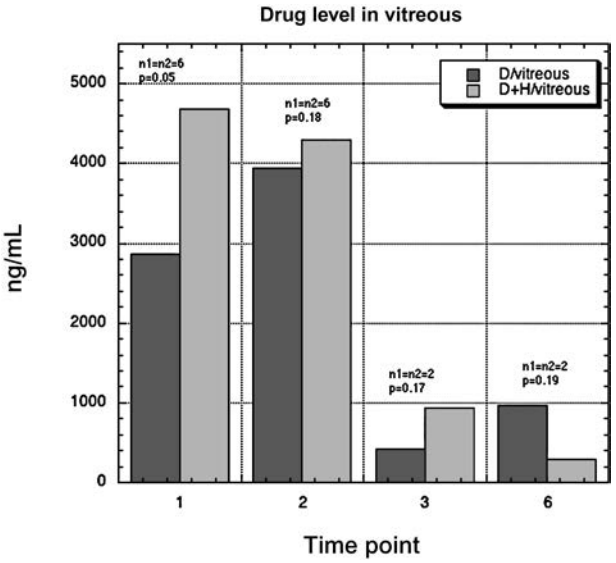

FIG. 7. The comparison of dexamethasone levels in the rabbit vitreous at different time points after sub-Tenon's injection of dexamethasone alone versus dexamethasone and rhuPH20.

CONCLUSIONS

In summary, sub-Tenon's coinjection of rhuPH20 with dexamethasone results in transient l increase in dexamethasone levels in ocular tissues and the serum, with significant increase detected in the choroid and the retina 3 h after administration. The clinical utility of this enzyme as a drug delivery enhancer may be limited to therapeutics, which would benefit from a short boost in their intraocular levels, such as tissue plasminogen-activator or viral vectors for gene therapy.

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Received: March 27, 2006
Accepted: May 31, 2006

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