Evaluation of the ocular penetration of topical alpha-luminol (Galavit®/GVT®)

Enry G. da Silva,* Juliet R. Gionfriddo,* Susan F. Hudachek,* Daniel L. Gustafson,* Francisco J. Olea-Popelka,* Virgina L. Scofield,† Cynthia C. Powell* and Ashley E. Hill*

*Department of Clinical Sciences, Colorado State University, College of Veterinary Medicine, Fort Collins, CO, USA; and †Department of Carcinogenesis, The University of Texas M.D. Anderson Cancer Center, Science Park-Research Division, Smithville, TX, USA

Address communications to:
J. R. Gionfriddo
Tel.: (970) 297-4173
Fax: (970) 297-1275
e-mail: juliet.gionfriddo@colostate.edu

Abstract

Purpose Oxidative stress plays a major role in the pathogenesis of many neurodegenerative diseases. It has also been implicated as part of the pathogenic mechanisms in the development of glaucoma. Alpha-luminol has shown profound anti-inflammatory and antioxidant effects in both experimental animal and human clinical studies. The purpose of this pilot study was to investigate for the first time the ocular penetration of topical alpha-luminol.

Methods Nine animals were divided into three treated groups (three animals each; one drop OU/n = 18), each group receiving a different concentration of the eyedrop (0.5%, 1.5%, 2.5%). Aqueous humor and peripheral blood samples were obtained from each rabbit at three different timepoints (20 min, 4 h and 12 h). Samples were analyzed by means of high performance liquid chromatography and mass spectrometry; median values were compared.

Results Alpha-luminol was found in the aqueous humor in all treated groups at all timepoints. At the 2nd and 3rd timepoints (4 h and 12 h), aqueous humor levels decreased significantly (P < 0.05) for two of the three dosages tested and it was not detectable in some eyes. The highest aqueous humor concentration of the drug was 272 ng/mL after 20 min (0.0217% of one drop, 2.5% group). Alpha-luminol was found in the vitreous in two animals, one in the 1.5% and another in the 2.5% group (16.4 and 21.5 ng/mL, respectively), at 12 h.

Conclusions Topically administered alpha-luminol readily penetrates into the anterior chamber and can penetrate into the vitreous chamber. Further investigation is warranted to better understand the intraocular pharmacokinetics of alpha-luminol.

Key Words: alpha-luminol, eye, Galavit®, glaucoma, GVT®, oxidative stress, topical

INTRODUCTION

Oxidative stress is thought to play an important role in retinal diseases such as diabetic retinopathy and glaucoma. Reactive oxygen species (ROS) may initiate and potentiate retinal damage. Thus, there has been much investigation into the use of antioxidant therapy to both prevent and ameliorate retinal damage in ROS-mediated retinal diseases. One of these anti-oxidants is alpha-luminol. Alpha-luminol is a refined monosodium luminol compound (monosodium 5-amino-2-3-dihydro-1-4-phthalazine dione) that was developed in the late 90s as part of a secret space research program at a military laboratory in Russia. It was originally used for human administration, supposedly to protect astronauts against radiation in space.12 Amongst the different names of the drug are GVT®, Galavit® and MSL® (monosodium alpha-luminol). Initial reports of the beneficial effects of alpha-luminol are found mainly in the Russian literature. Several abstracts on clinical evaluations of alpha-luminol were presented during conferences in Russia, although none of these initial studies were published in peer-reviewed journals. Alpha-luminol was once thought to be a ‘miracle drug’ as well as a cure for cancer, concepts that were later shown to be false. Further investigations on the efficacy and therapeutic properties of alpha-luminol have recently been published in more widely accessible journals.

Alpha-luminol has been shown to have profound anti-inflammatory and immunomodulatory effects in humans and laboratory animals without any deleterious effects.
The immunomodulatory effects of alpha-luminol have been linked to a ‘reliable’ increase in T-helper (CD4+) and natural killer cell (CD16+) counts in non cancer human patients, and mild increases in the concentrations of interleukin-1 (IL-1) and tumor necrosis factor (TNF) in mice. In a study in mice experimentally infected with the cytopathic retrovirus ts1, alpha-luminol delayed the development of immunodeficiency, neuropathology, and the clinical signs of neuromuscular degeneration that were present in the controls. Additionally, it is a potent antioxidant and one of the proposed mechanisms for its antioxidant properties is the up-regulation of cellular nuclear factor erythroid 2-related factor 2 (Nrf-2) levels.

Nuclear factor erythroid 2-related factor 2 is a cytoplasmic transcription factor required for the activation of the antioxidant responsive element (ARE). The Nrf2-ARE pathway is responsible for the activation of protective genes and detoxification enzymes, which are essential in fighting oxidative stress and toxic changes. In previous investigations into the possible medical uses for alpha-luminol it was given to test animals via parental routes (e.g. oral, intraperitoneal). In order to target intraocular structures (such as the lens, uveal tract and retina) for drug therapy, the parenteral route is often used. However, a topical drug that is effective in reaching these structures in therapeutic concentrations would be preferable because topical drops provide for more targeted therapy, are easier to administer and lower amounts of the drug are absorbed systemically. The objective of this paper is to evaluate the ocular penetration of topical alpha-luminol and describe the possible mechanisms of action of the drug.

MATERIAL AND METHODS

Drug formulation

The eyedrops containing alpha-luminol were formulated and donated for this study by Bach Pharma, North Andover MA. The 0.5%, 1.5% and 2.5% concentration eyedrop vials were prepared from a powder of alpha-luminol and sterile NaCl. Commercially available eye droppers with a drop size of 50 μL were used to administer the drug. This drop size did not result in spill-over of tears.

Animals

Experiments were carried out in 11 purpose-bred, adult, New Zealand White rabbits (five males and six females) weighing an average of 2.9 kg. This study was performed in accordance with the Colorado State University Animal Care and Use Committee. Animals were acclimated for 1 week prior to the initiation of the study, after which an ocular exam was performed in each rabbit in order to rule out previous or ongoing ocular problems.

Rabbits were divided into four groups, three treatment groups composed of three animals each and one control group of two animals (n = 22 eyes). Treatment groups were tested using topical alpha-luminol in concentrations of 0.5%, 1.5% and 2.5% eyedrops. The control group received topical NaCl. All treated animals received a single drop simultaneously in both eyes.

Tissue collection

Aqueous humor and peripheral blood samples were taken from each animal at three different, randomly chosen timepoints: 20 min, 4 h and 12 h post treatment. At each timepoint, the examiner (EG) looked for potential signs of ocular toxicity from the topical compound (increased ocular discharge, abnormal degree of conjunctival hyperemia, blepharospasm, pain, peri-ocular pruritus, reluctance to touch, vocalization). Aqueous humor samples were obtained after a limbal paracentesis, following topical and local anesthesia with 0.5% proparacaine hydrochloride. At the last timepoint, rabbits were humanely euthanized with anesthetic overdose intravenously. Both eyes of each rabbit were enucleated. The cornea was removed at the level of the limbus, and after the iris was sectioned and removed, the lens was collected. Vitreous was carefully dissected posteriorly and removed as a whole, followed by the neuro-retina. All samples were placed in sterile tubes and stored under −80 °C for later evaluation. Quantification of alpha-luminol was done by high performance liquid chromatography (HPLC) coupled to electrospray ionization (ESI) mass spectrometry (MS).

HPLC apparatus and conditions

The HPLC system consisted of an Agilent 1200 Series binary pump SL, vacuum degasser, thermostatted column compartment SL (Agilent Technologies, Santa Clara, CA, USA) and a CTC Analytics HTC PAL System autosampler (Leap Technologies, Carrboro, NC, USA). The HPLC column was a Waters XBridge Phenyl column (4.6 × 50 mm ID, 5.0 μm bead size) (Waters Corporation, Milford, MA, USA) protected by a SecurityGuard™ C18 cartridge (4 × 2.0 mm ID) (Phenomenex, Torrance, CA, USA) and maintained at a temperature of 40 °C. The mobile phase consisted of 99% methanol and 1% 0.1% formic acid in water. The system operated at a flow-rate of 1 mL/min.

MS apparatus and conditions

Mass spectrometric detection was performed on an API 3200™ triple quadrupole instrument (Applied Biosystems Inc, Foster City, CA, USA) using multiple reaction monitoring. Ions were generated in positive ionization mode using an electrospray interface. Compound-dependent parameters were as follows: declustering potential (DP): 44 V; entrance potential (EP): 4 V; collision cell entrance potential (CEP): 15.127 V; collision-activated dissociation (CAD) gas (nitrogen): 6 psi; collision energy (CE): 30 V and collision cell exit potential (CXP): 2.5 V. Source-dependent parameters were as follows: nebulizer gas (GS1): 40 psi; auxiliary (turbo) gas (GS2): 60 psi; turbo gas temperature (TEM): 550 °C; curtain gas (CUR): 50 psi, ionspray voltage (IS): 4500 V and interface heater (IH): 100 °C. Peak areas obtained from
MRM of alpha-luminol (m/z 178 → 105) were used for quantification.

Sample preparation

Standard solutions containing alpha-luminol were prepared in water. The drug was extracted from rabbit serum and ocular tissues (aqueous humor, vitreous humor, lens and retina) using Waters Oasis® HLB 1cc (30 mg) solid phase extraction cartridges (Waters Corporation, Milford, MA, USA) following the manufacturer’s instructions for the generic SPE method. Briefly, cartridges were conditioned with 1 mL of methanol, equilibrated with 1 mL of water, loaded with sample, washed twice with 1 mL of water and eluted with 1 mL of methanol. For serum, aqueous humor and vitreous humor, 100 µL of each liquid was loaded onto the cartridge for extraction. The lens was weighed, the appropriate volume of water was added to result in a tissue concentration of 500 mg/mL and the sample was sonicated on ice until the tissue was homogenated. For the retina, 250 µL of water was added to the entire tissue and the sample was sonicated on ice until the tissue was homogenated. For both the lens and the retina, 100 µL of the homogenate was loaded onto the cartridge for extraction. An aliquot of 50 µL of the methanol eluant was injected into the LC/MS system for analysis.

Statistical analysis

For each time point (20 min, 4 h, and 12 h post administration), the amount of drug in the aqueous humor using 0.5% drops was compared to that using 1.5% and 2.5% drops using Wilcoxon rank-sum testing. Similarly, for each dosage group (0.5%, 1.5%, and 2.5% drops), the amount of drug in the aqueous humor at 20 min was compared to that at 4 and 12 h post administration using Wilcoxon rank-sum testing. For all tests, results were considered statistically significant at P ≤ 0.05.

RESULTS

Peripheral blood

After single-dose administration of 0.5% alpha-luminol eyedrops, the drug was not detected in the peripheral blood at any timepoint (level of detection ≥ 0.02 ng/mL) nor was it detected at 12 h in either the 1.5% or 2.5% groups. However, it was detectable at 20 min in one rabbit from the 1.5% group (18.8 ng/mL), in two rabbits from the 2.5% group (21.4 ng/mL, 22.2 ng/mL); also after 4 h in two rabbits, one from the 1.5% group (11.6 ng/mL) and one from the 2.5% group (35.8 ng/mL).

Aqueous Humor

After single-dose administration of 0.5%, 1.5% and 2.5% alpha-luminol eyedrops, the amount of drug in the aqueous humor per eye ranged from undetected levels to 272 ng/mL. At 20 min after administration, the level of alpha-luminol in the aqueous humor of rabbits administered 1.5% drops was significantly higher than rabbits administered 0.5% drops (Table 1). No other significant differences due to dosage were observed (Table 1). In rabbits administered 0.5% drops and 1.5% drops, aqueous humor levels of alpha-luminol were significantly lower at 4 and 12 h post-administration compared to 20 min post-administration (Table 2). In rabbits administered 2.5% drops, aqueous humor levels of alpha-luminol were significantly lower at 4 h after administration than 20 min after administration, but were not significantly different 12 h after administration (Table 2). No drug was found in rabbits from the control group. Raw values from the peripheral blood and aqueous humor are shown in Table 3.

Vitreous humor, retina and lens

After single-dose administration of 0.5%, 1.5% and 2.5% alpha-luminol eyedrops, the vitreous humor concentrations were below the level of detection (0.02 ng/mL) in most of the animals, with exception of one rabbit in group 2 (1.5%) (16.4 ng/mL) and one rabbit in group 3 (2.5%) (21.5 ng/mL). No drug was detected in either the retinal homogenate or the lens.

DISCUSSION

In this study we aimed to characterize the penetration of topically administered alpha-luminol into various ocular compartments using a rabbit model. Our results demonstrate that the topically administered, potent antioxidant alpha-luminol readily penetrates the cornea and anterior chamber. In this formulation, it did not cause ocular irritation or local toxicity, as none of the rabbits showed signs of blepharospasm, itching or conjunctival hyperemia, during the study.

The drug concentrations in the aqueous humor were relatively low. The highest concentration achieved in any rabbit was 272 µg/mL at the 20 min timepoint. However, this was only 0.02% of the drug contained in one drop. Whether this

<table>
<thead>
<tr>
<th>Time point</th>
<th>0.5% median</th>
<th>1.5% median (P-value)</th>
<th>2.5% median (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 min</td>
<td>39.8</td>
<td>131.5 (0.04)</td>
<td>166.5 (0.15)</td>
</tr>
<tr>
<td>4 h</td>
<td>&lt;0.02</td>
<td>15 (0.10)</td>
<td>20.8 (0.10)</td>
</tr>
<tr>
<td>12 h</td>
<td>&lt;0.02</td>
<td>&lt;0.02 (0.90)</td>
<td>12.5 (0.15)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dosage (drops)</th>
<th>20 min median</th>
<th>4 h median (P-value)</th>
<th>12 h median (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5%</td>
<td>39.8</td>
<td>&lt;0.02 (0.03)</td>
<td>&gt;0.02 (&lt;0.01)</td>
</tr>
<tr>
<td>1.5%</td>
<td>131.5</td>
<td>15 (&lt;0.01)</td>
<td>&lt;0.02 (&lt;0.01)</td>
</tr>
<tr>
<td>2.5%</td>
<td>166.5</td>
<td>20.8 (0.05)</td>
<td>12.5 (0.17)</td>
</tr>
</tbody>
</table>
The amount of drug is therapeutically relevant remains to be investigated. Interestingly, this rabbit with the highest drug concentration was part of the 2.5% group and was one of the few rabbits in which the drug was also found in the serum. Detectable serum concentrations in this rabbit were found only in the first serum timepoint (22.2 ng/mL).

There were important differences in the amounts of drug present in the aqueous humor in the lowest treatment concentration group (group 1, 0.5%) vs. group 2 (1.5%) (diff = +91.7 ng/mL) and group 3 (2.5%) (diff = +126.7 ng/mL) at the first timepoint. These results suggest that both 1.5% and 2.5% topical alpha-luminol drops were able to achieve aqueous humor levels well above those found in group 1 (0.5%). Despite the lack of significance at the 5% level (95% confidence interval, \( P < 0.05 \)), the results are promising considering that even with such a small sample size, a borderline significant difference at the 10–15% level (85–90% confidence interval, \( P \leq 0.15 \)) was detected when comparing median levels of alpha-luminol in eyes from rabbits between the 0.5% group and the 1.5% and 2.5% group. This suggests that increasing the concentration of the drug in the eyedrops could potentially produce therapeutically significant intraocular levels. In rabbits administered 2.5% drops, aqueous humor levels of alpha luminol were significantly lower at 4 h after administration than 20 min after administration, but were not significantly different 12 h after administration (Table 2). This is due to an exceedingly high aqueous humor value (730 ng/mL) in group 3, at the 12 h period, which was most likely a lab error (Table 3).

Rabbits were chosen for this study because their corneal structure and aqueous humor volume are similar to that of humans.\(^{28–30}\) In addition, they have a small body size, are easy to handle and have large eyes.\(^{30,31}\) However, rabbits have an especially labile blood–aqueous barrier (BAB)\(^{31}\) and this could have led to leakage of serum-derived alpha-luminol into the eye after the first paracentesis. Therefore, the aqueous humor drug levels obtained at the first paracentesis may represent the best indicators of the true ocular penetration properties of the drug. Moreover, since in most of the rabbits serum levels were either not detectable or lower than the aqueous humor levels, it is unlikely that the drug levels present inside the eye at the later timepoints were attributable to serum leakage.

A major limitation of this study was the small sample size (three rabbits per group, \( n = 6 \) eyes in each treatment group), which can also account for the high data variability present in the study. However, this was designed as a pilot study and the data was analyzed by statistical techniques that were appropriate for the small sample size.

The significant decrease in intra-ocular drug concentrations from the first timepoint is expected in any ocular pharmacological study. The fact that two of the rabbits

<table>
<thead>
<tr>
<th>Alpha-luminol drop dose</th>
<th>Rabbit no.</th>
<th>Peripheral blood (ng/mL)</th>
<th>Aqueous humor (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20 min</td>
<td>4 h</td>
</tr>
<tr>
<td>0.50%</td>
<td>1</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>M’</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1.50%</td>
<td>4</td>
<td>18.8</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>6.27</td>
<td>3.87</td>
</tr>
<tr>
<td></td>
<td>M’</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>10.84</td>
<td>6.69</td>
</tr>
<tr>
<td>2.50%</td>
<td>7</td>
<td>21.4</td>
<td>35.8</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>22.2</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>14.53</td>
<td>11.94</td>
</tr>
<tr>
<td></td>
<td>M’</td>
<td>21.4</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>12.58</td>
<td>20.66</td>
</tr>
</tbody>
</table>

M, mean; M’, median, SD, standard deviation.

*Indicates an outlier due to lab error.
showed an increase in the alpha-luminol concentration with time (rab1-OD, rab8-OD; Table 3) could not be explained). The aqueous and vitreous humor turnover rate may profoundly affect the pharmacokinetics of a drug. The aqueous humor turnover rate in rabbits (half life = 46 min) is similar to that of humans (half life = 52 min) which is relatively slow compared to the fast turnover rate of the aqueous portion of the vitreous. From experiments in which heavy water was injected intraperitoneally and subsequently detected in the vitreous, it was calculated that 50% of the water content of the vitreous is replaced every 10–15 min and 75–87% is replaced in 30 min. Consequently, the fact that we were able to find the drug in the vitreous after 12 h in two rabbits may mean that it could have been found in higher concentrations had the vitreous been sampled at earlier timepoints.

Topical alpha-luminol is largely hydrophilic. Drugs with higher lipophilicity have a greater ability to penetrate an intact cornea. Future pharmacodynamic studies may consider increasing the penetration potential of the drug in order to achieve constant adequate intraocular concentration; potential periocular routes for posterior segment drug delivery should also be investigated, such as sub-conjunctival and sub-tenon routes. The importance of achieving therapeutic levels of alpha-luminol in the retina stems from its potential as a strong preventive and therapeutic drug in this structure. In the central nervous system as well as the retina, glial cells protect, up-regulate and stabilize neuronal levels of Nrf2.

The Nrf2 pathway in the neuronal cells coordinates the up-regulation of antioxidant defenses and confers protection to neighboring neurons. This protective mechanism against oxidative stress is up-regulated in neuroimmunodegenerative processes such as in Alzheimer’s, Parkinson’s and Huntington’s disease. In diseased neurologic tissue, therapeutically increasing the expression of Nrf2 in glial cells significantly delays the onset of the disease and extends neuronal survival, both in vitro and in vivo.

The biochemical composition of ocular structures, especially that of the retina (high concentration of unsaturated fatty acids in the retina), is an important factor that makes the eye more susceptible to oxidative stress, in comparison to other organs. Oxidative stress-induced changes and microvascular damage occur in retinas of dogs with primary glaucoma. This was demonstrated by documenting the loss of major retinal antioxidants such as glutathione and taurine and changes in retinal glutamate in these animals. In the DBA2J mouse model of glaucoma, administration of oral alpha-luminol decreased oxidative stress and reduced changes in glutamate distribution in their retinas. This suggests that alpha-luminol could be used to reduce retinal oxidative stress and consequent neuronal damage in canine glaucoma as well. Further pharmacokinetic data is necessary along with the determination of therapeutic drug levels in the retina, prior to the clinical use of alpha-luminol in glaucoma. This pilot study represents the first step in this process as it investigates the use and the ocular pharmacokinetics of topical alpha-luminol for the first time.

ACKNOWLEDGMENTS

Supported by a grant from the Longevity Foundation, Austin, TX. The authors would like to thank Lauren Marchewitz for the help in acquiring the data for this study.

REFERENCES


