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Ocular biodistribution of cysteamine delivered by a sustained release microsphere/thermoresponsive gel eyedrop

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ABSTRACT

The objective of the investigation was to determine the ocular biodistribution of cysteamine, a reducing agent used for treatment of cystine crystals in cystinosis, following topical administration of a sustained release formulation and traditional eyedrop formulation. To the right eye only, rabbits received a 50 μL drop of 0.44% cysteamine eyedrops at one drop per waking hour for 2, 6, 12, and 24 h. A second group received one 100 μL drop of a sustained release formulation containing encapsulated cysteamine microspheres suspended in a thermoresponsive gel. Upon serial sacrifice, ocular tissues from both eyes and plasma were obtained and quantified for cysteamine using LC-MS/MS. Cysteamine was detected in the cornea, aqueous humor and vitreous humor. Systemic plasma concentrations of cysteamine from treatment groups were below the limit of detection. As expected, 0.44% cysteamine eyedrops when administered hourly maintained drug concentrations within the cornea at a magnitude 5 times higher than a single dose of the sustained release formulation over 12 h. The sustained release formulation maintained cysteamine presentation across 12 h from a single drop. These studies demonstrate distribution of cysteamine to the eye following topical administration, including high drug uptake to the cornea and low systemic distribution.

1. Introduction

Cystinosis is an inherited rare disease that occurs in 1 in 100,000–200,0000 people worldwide (Bertholet-Thomas et al., 2017). Patients with cystinosis exhibit high accumulation of cystine crystals in all organ tissues resulting in systemic health complications that progress with age. In the eye, cystine crystals are most notably present in the cornea as spindle-like structures that are hyper-reflective to visible light. Patients experience severe light sensitivity from corneal cystine crystals which adds vision complications to a burdensome, life-long disease (Gahl et al., 2000; Kaiser-Kupfer et al., 1987; Tsilou et al., 2003). Furthermore, recurrent corneal epithelial erosion occurs when treatment with traditional eyedrop regiments is not effective. Cysteamine hydrochloride is the salt form of an aminothiol pharmacologic compound approved for the reduction of corneal cystine crystals in cystinosis. An aqueous eyedrop formulation containing 0.44% cysteamine (Cystaran®, Leadiant Biosciences, Inc.) was the first such FDA-approved therapy, commonly prescribed for one drop every waking hour, about 6–12 drops per day (Huynh et al., 2013). After opening, the shelf life of cysteamine eyedrops is one week under refrigeration due to the oxidative instability of cysteamine. A gel formulation approved in the EU containing 5.5 mg cysteamine hydrochloride, equivalent to 0.35% cysteamine (Cystadrops®), gained FDA approval in 2020. This viscous gel formulation contains carboxymethyl cellulose to reduce the frequency of administration when prescribed at 4 drops per day and has a similar shelf life of one week under 4–25 °C. ((Lyseng-Williamson, 2017), "U.S. FDA Approves CYSTADROPS" 2021). Cysteamine is effective by entering the cornea and undergoing redox reactions

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intracellularly to serve as a cystine depleting agent to lower the cystine content of cells (Gahl et al., 2002).

Upon instillation of Cystaran®, patients may experience significant adverse effects lasting up to an hour which include redness, stinging, and burning (Huynh et al., 2013). In addition to transient irritation, when patients received Cystadrops®, some experienced sticky eyes and eyelashes as reported in open-label comparative phase III clinical trials (Liang et al., 2017). Regardless of adverse effects, patients were appreciative of a gel formulation that reduced the dosing frequency of traditional cysteamine eyedrops from 6 to 12 drops per day to only 4 drops per day, based on qualitative surveys from clinical trials (Labbé et al., 2014; Liang et al., 2017). The introduction of any alternative formulation is encouraging for patients at a time when the supply of Cystaran® eyedrops suffered from a prolonged medication shortage starting in October 2020 due to bankruptcy of its independent manufacturer (COVID-19, 2022). This has constrained access to cysteamine requiring patients to source eyedrops from specialty pharmacies and highlighted the need for additional treatment options. Cystaran® is expected to once again be available in April 2022, yet priority must continue to be placed on developing ocular cysteamine formulations that improve drug stability and reduce dosing frequency.

A significant challenge to reformulating cysteamine is the compound's high oxidative instability resulting in an inactive dimer, cystamine. The eyedrop formulations may be frozen upon receipt and stored under refrigeration until use, however, they must not undergo repeated freeze–thaw cycles and are to be discarded after one week (Huynh et al., 2013; Gahl et al., 2002). The repeated opening of a bottle likely introduces oxygen species that promote further degradation of cysteamine to cystamine. Researchers explored this concept by adding oxygen-resistant hydrophobic layers into an eyedrop bottle to limit the effect of ambient air entering the bottle, resulting in longer storage times (although effects of the additives on bioactivity were not tested) (Dixon et al., 2018). Other research has focused on methods to reformulate cysteamine into corneal drug delivery systems to improve drug stability and ocular bioavailability.

Experimental cysteamine drug delivery systems include polymeric vehicles such as viscous gels (Buchan et al., 2010), hydrogels (Luaces-Rodríguez et al., 2017), contact lenses with vitamin e (Hsu et al., 2013), and a dissolvable thin film nanowafer (Marcano et al., 2016). Of the current experimental formulations, the nanowafer reduced the dosage to once daily in the cystinosis rodent model and improved the stability of cysteamine to four months at room temperature under closed-storage conditions (Marcano et al., 2016). Beyond pharmacological approaches to treatment, systemic transplantation of hematopoietic stem progenitor cells rescued corneal defects in the rodent model of cystinosis (Rocca et al., 2015).

Recently, our group has developed and tested a combined thermoresponsive gel/microsphere eyedrop containing encapsulated cysteamine (Jimenez et al., 2021). Spray-dried cysteamine microspheres are loaded into a poly (N-isopropylacrylamide) based gel (SD-CMS/Gel) and release cysteamine *in vitro* for 24 h in a sustained manner. The SD-CMS/Gel can be administered similarly to a traditional eye drop, but forms a pliable, nondegradable depot after exposure to ocular surface and conjunctival temperatures (e.g, 32–34 °C) in the lower fornix of the eye. This was verified *in vivo* by testing for safety and retention over 24 h in an ophthalmic animal model. Prior to *in vivo* studies, the delivery system was confirmed to be non-irritating in *ex vivo* organotypic models and further tolerated well *in vivo*. The stability of cysteamine in microspheres was extended to 7-weeks utilizing cysteamine eyedrops as a control for comparison proton nuclear magnetic resonance studies. The characterization of our formulation and testing *in vivo* supported evidence towards translation into ocular biodistribution studies, which the current research field lacks.

Herein, we report the first large animal ocular distribution study of cysteamine after administration of topically applied eyedrop formulations. To our knowledge, the current FDA-approved formulations have no available ocular pharmacokinetic or biodistribution data, which further contributes to the complexity of cysteamine eyedrop reformulation. We hypothesized that cysteamine presentation would be comparable in the SD-CMS/Gel sustained release formulation at a far lower dosing rate (once daily versus hourly). The maximum concentration of cysteamine from the gel was measured between 2 and 6 h, whereas that of the eyedrop was measured within 2 h. The biodistribution data confirmed uptake in the cornea and aqueous humor, supporting evidence for transscleral permeation from both SD-CMS/Gel and traditional eyedrops. Plasma concentration levels remained below detection limits indicating low systemic biodistribution. The results suggest significantly improved stability and enhanced delivery *in vivo* from SD-CMS/Gel, which supports the continued investigation of this novel platform for treating corneal cystinosis. These studies also provide a framework for future large animal studies testing reformulated cysteamine or other small molecule ophthalmic drugs into long-term drug delivery systems.

2. Materials and methods

2.1. Ophthalmic model description

Animal subjects were utilized for this research following the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the University of Pittsburgh Institutional Animal Care and Use Committee. These guidelines follow the National Research Council's Guide for the Care and Use of Laboratory Animals. Four-month-old, mixed sexes, New Zealand white rabbits were purchased from Envigo (Somerset, NJ, USA). Prior to experiments, the nictitating membrane of the right eye (OD) of each subject was resected according to previously published methods (Jimenez et al., 2021; Mammen et al., 2016; Fedorchak et al., 2017). Briefly, under systemic anesthesia (10 mg/kg of ketamine (Ketathesia; Henry Schein Animal Health, Dublin, OH, USA) and 1 mg/kg of xylazine (AnaSed Injection; Lloyd Laborato ries, Shenandoah, IA, USA)), one topical eyedrop of 0.5% proparacaine (Baush + Lomb, Bridgewater, NJ, USA) was administered to the ocular surface. Then, the nictitating membrane was surgically removed with a scalpel and cauterized. Following the procedure, one topical eyedrop of 0.3% tobramycin (Bausch + Lomb, Bridgewater, NJ, USA), and one topical eyedrop 1% prednisolone acetate (Pacifc Pharmaceuticals Inc., Rancho Cucamonga, CA, USA) was immediately administered and repeated once daily for the following 4 days to prevent infection and manage inflammation. The eyes of rabbits were examined using slit-lamp photography (Eyephotodoc, Fullerton, CA, USA) throughout the study. After 7 days, baseline photography and baseline intraocular pressure (IOP) measured with tonometry (Tonovet Plus, Icare, Finland) were captured. A pilot study investigating the safety of instillation technique and materials was carried out prior to implementing the larger biodistribution study. That study utilized four timepoints (2, 6, 12, and 24 hr) to evaluate animals randomized to one of two groups (N = 3 per group, mixed sexes). A timeline of our study design and sample size is represented in Fig. 1.

2.2. Cysteamine formulations and material characterization

Cysteamine eyedrops were prepared according to the Cystaran® product insert (Cystaran_PI.Pdf." Accessed September 5, 2018) by mixing cysteamine hydrochloride (66 mg) in 15 mL deionized water with 0.01% benzalkonium chloride (1.5 mg) and 0.90% sodium chloride (135 mg). A pH of 4.0 – 4.5 was achieved by titrating 0.1 N hydrochloric acid and 0.1 N sodium hydroxide, approximately 10 μL at a time. The cysteamine solution was aliquoted into 1 mL volumes in 2.5 mL amber vials and bubbled with nitrogen gas in a glovebox (Fischer Scientific, Pittsburgh, PA, USA). Samples were stored frozen at −20 °C and wrapped in Parafilm until use. Any unused cysteamine solution was immediately discarded within 24 hr of unsealing.

The sustained release formulation, SD-CMS/Gel, was prepared using

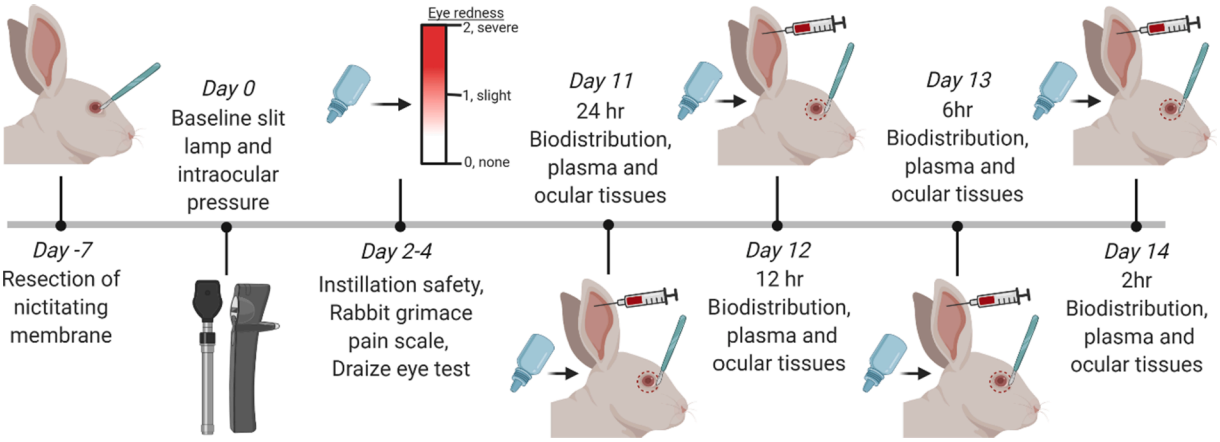

Fig. 1. A timeline for the experimental rabbit model used for ocular biodistribution of cysteamine delivered from topical eyedrop formulations. Resection of nictating membrane, the third eyelid, was performed seven days prior to baseline safety measurements. Graphic was created using Biorender.com.

our previously published method (Jimenez et al., 2021). Briefly, 2 g of cysteamine hydrochloride and 8 g of poly(DL-lactide-co-glycolide) (IV 0.14–0.22 dl/g, Mw: 4000–15,000) (Evonik Maryland, USA) in a cosolvent consisting of a methanol:dichloromethane (10:90,v/v) solution was mixed and fed to a mini spray dryer (Büchi, New Castle, Delaware, USA). Cysteamine free microspheres (SD-BLANK-CMS) were produced using the same fabrication process without the addition of cysteamine hydrochloride. The pNIPAAm based gel was fabricated using free radical polymerization with the addition of poly(ethylene glycol) (PEG 200 kDa). Suspensions of SD-CMS/Gel and SD-BLANK-MS/Gel were fabricated by mixing 10 mg of SD-CMS to 100 μL of Gel. Cysteamine within 10 mg of SD-CMS is equivalent to 2.6 mg of cysteamine as determined in previous studies (Jimenez et al., 2021) All SD-CMS/Gel and SD-BLANK-MS/Gel samples were suspended immediately before administration to subjects. For the present study, scanning electron microscopy (SEM) was utilized to obtain images of SD-CMS/Gel prior to administration (0 hr) and after administration (24 hr) of the delivery system. Samples were processed by freeze drying in a 1 mL syringe under liquid nitrogen. After drying, a razor blade was used to cut cross-sections. Cross sections were mounted on stubs with mounting tape and gold sputtered prior to SEM imaging.

2.3. Instillation safety pilot study

Semiquantitative Draize eye test scores (OECD 2021) and rabbit grimace scores (Keating et al., 2012; Hampshire and Robertson, 2015) were used to evaluate possible adverse effects at instillation. Subjects were randomly organized into three groups, where each group received one dose of a given formulation: 50 μL of a cysteamine eyedrop (N = 3), 100 μL of SD-BLANK-CMS/Gel or 100 μL of SD-CMS/Gel. Rabbits were gently restrained (no anesthesia given) using the towel wrapped "burrito" method (Varga, 2014) and given the appropriate eyedrop formulation. Slit lamp images were captured at the following timepoints: 0 mins, 0.5 mins, 1 min, 5 min, 10 min, 15 min, 30 min, and 1 hr. Images were de-identified and scored in a masked fashion by an ophthalmologist. Draize eye test scores included scoring for opacity in the cornea (0,1,2,3,4), iris hemorrhage (0,1,2) Conjunctiva redness (0, 1, 2, 3), and chemosis (i.e., swelling, 0, 1, 2, 3, 4) (OECD, 2021). The rabbit grimace scores for orbital tightening as indicated by closure of the eyelid was scored at the following levels: not present (0), moderately present (1), and obviously present (2) (Hampshire and Robertson, 2015).

2.4. Treatment timepoints and tissue collection

Rabbits were randomly assigned into one of two groups based on treatment formulations. Each time point (2, 6, 12, 24 hr) consisted of one group receiving cysteamine eyedrops (N = 3, total 12 rabbits) and one group receiving SD-CMS/Gel (N = 3, total 12 rabbits). Each treatment group was run in parallel for each timepoint where the cysteamine eyedrops received one drop per hour (e.g. 2 hr received 2 eyedrops on the hour). For the cysteamine eyedrop group, the last dose was given at 12 hr for the 24 hr timepoint.to simulate the recommended dosing frequency in patients, i.e. no drops were administered between hours 12–24. The SD-CMS/Gel group received a single dose regardless of the endpoint for a given rabbit. At predetermined timepoints, a single sample of 1 mL of whole blood from each subject was placed into heparinized glass tubes. Then, a lethal dose of ketamine/xylazine was administered via marginal ear vein. Immediately after euthanizing, both eyes were enucleated from subjects and placed on dry ice. While frozen, the cornea was excised to access aqueous humor and vitreous humor. These fluids were distinguishable visually with reference to the lens and trabecular meshwork where the aqueous humor is anteriorly and vitreous humor resides posteriorly of these structures. Additionally, at sacrifice, eyelid tissue from both eyes were shaved and exenterated using iris scissors. Tissue samples were washed in PBS pH 7.4 for 1 hr. Then, eyelid tissues were placed in 10% formalin and fixed for 48 hr at 4 °C then stored in a holding solution composed of 70% ethanol at 4 °C for 1 week. Samples were embedded in paraffin. Cross sections of the eyelid (stood up on its side to observe layers of eyelid skin) were stained with hemoxylin and eosin (H&E) and Verhoeff Van Gieson (VVG). Images were digitally archived and reviewed by a clinical ophthalmologist. Any morphological changes were observed and recorded.

2.5. Plasma, aqueous humor and vitreous humor cysteamine measurement with LC-MS/MS

To 80 μL of freshly collected whole blood, 20 μL of 150 mM N-ethylmaleimide (NEM) in deionized water was immediately added and mixed, a critical step in preventing artificial oxidation of a number of thiol containing blood metabolites (Sutton et al., 2018). The reaction was performed at room temperature for 30 min. Then, samples were stored at −80 °C until cysteamine extraction for LC-MS/MS analysis. For aqueous humor and vitreous humor, 80 μL of each was treated similarly by adding NEM and reacting for 30 min and storing at −80 °C. Upon extraction for MS, samples were thawed and extracted with 800 μL of cold (-20 °C) extraction solution of 90% acetonitrile/1% formic acid containing an internal standard of 1 μM NEM-d_4-cysteamine, (d_4-cysteamine HCL purchased from CDN Isotopes, Pointe-Claire, Quebec, CA). Samples were centrifuged at 4 °C at 16 kg. The supernatant was collected and placed into a new microtube and stored at − 80 °C. Samples were shipped on dry ice and sent to Clarus Analytical LLC (San Diego, CA), and diluted 3-fold in 80% methanol/0.1% formic acid, prior

to injection (5 μL) onto the LC-MS/MS.

The LC-MS/MS method was adapted from previously published method (Dohil et al., 2014) though using a different manufacturer's amide column (X-Bridge amide column, 2.1 mm × 150 mm, 3.5 μM particle size, Waters Corp. Milford, MA, USA) and instead monitoring the NEM-derivatized drug. An API 4500 triple quadrupole mass spectrometer (Sciex, Framingham, MA, USA) coupled to a Shimadzu Prominence FPLC (Shimadzu Corp. Kyoto, JPN) and HTC PAL autosampler (Trajan Scientific and Medical, Victoria, AUS) was used for data collection. A 5-minute isocratic LC method was used to monitor NEM-labeled cysteamine and NEM-d4-cysteamine, using a mobile phase consisting of: 80/20 acetonitrile/water, with 0.1% formic acid and 2 mM ammonium formate, column temperature set to 45 °C, and a flow rate of 0.4 mL/min. The following MRM transitions were used for quantification and qualification respectively: NEM-cysteamine (203⟩ 186 and 203 > 126) and NEM-d4-cysteamine (207⟩ 190). Collision energy was set to 18 V for MRM1, and 20 V for MRM 2. Other mass spectrometry parameters include: Declustering potential (55 V), EP/CXP (10 V/10 V), CUR (40), CAD (9), Ion spray voltage (5500), Source temp (450 °C), GS1 (40), and GS2 (70). A 9-pt calibration curve, using analyte depleted sera as the blank matrix (10 μL of each calibrator stock added to 90 μL of sera), were prepared with each batch, with linear regression fits showing correlation coefficients > 0.995. Three QC levels, 37 nM, 400 nM, and 4 μM, each analyzed with triplicates per batch, were evaluated for accuracy and precision (%CV), and all demonstrated accuracies between 85 and 115%, with CV < 15% at each level. This range (37 nM to 4 μM) represented the validated analytical measurement range for the assay, The limit of detection was determined to be 15 nM (~3 signal/noise). Values below this concentration were not reported. The true LLOQ of the assay was not determined, but based on back calculated accuracies of the lowest calibration points, it is likely somewhere between the LOD (15 nM) and the lowest QC level (37 nM) tested validated. Area ratios (endogenous cysteamine/d4-labeled) of samples were back-calculated to the calibration curves for determining moles of cysteamine using Multiquant software (Sciex, Framingham, MA, USA). In addition to validating accuracy and precision, spike recovery tests (performed in triplicate for the 3 QC levels mentioned above), was determined to have an average value of 92% +/- 7%. Other aspects of method validation such as specificity/selectivity, matrix effect, and carryover were also evaluated. No interferences were found in biological samples lacking cysteamine, and negligible ionic suppression was detected due to adequate sample dilution and sufficient chromatographic retention of the analytes of interest.

2.6. Corneal tissue cysteamine measurement with LC-MS/MS

Flash frozen corneal tissue was excised from the globe of harvested eyes. Corneal tissue was cryopulverized in liquid nitrogen with a stainless steel pulverizer (BioSpec Products, Inc. Bartlesville, OK, USA). Corneal powder from cryopulverization was weighed into 10 mg-25 mg aliquots and placed into cold, pre-weighed microtubes. To tissue aliquot, 200 μL of 30 mM NEM in deionized water was added and mixed. The reaction was performed at room temperature for 30 min. Then, samples were stored at −80 °C until cysteamine extraction for MS. For extraction, thawed samples received 1 mL of cold (-20 °C) extraction solution of 95% acetonitrile/1% formic acid containing an internal standard of 1 μM NEM-d_4-cysteamine. Samples were then placed into lysing matrices containing garnet beads and homogenized in a cold room (approximately 4 °C) using a FastPrep 24 homogenizer (MP Biomedicals, Solon, Ohio, USA). Homogenized samples were placed in −20 °C for 1 hr. Then, samples were centrifuged at 4 °C at 16 kg. The supernatant was collected and placed into a new microtube and stored at − 80 °C. Cysteamine levels were analyzed as indicated by the aforementioned MS methods and normalized based on the corneal mass prior to cryopulverization.

2.7. Statistical analyses

The means and standard deviations of N = 3 samples for intraocular pressure (IOP), rabbit grimace scores, and Draize eye scores were reported. For the IOP of rabbits, a one-way, repeated measures ANOVA and a Tukey's Multiple Comparison test was performed to compare each timepoint between the treatment groups (four treatments). For rabbit grimace testing, a Man Whitney *U* test was performed to compare clinical scores between four treatment groups. The concentration of cysteamine in tissues and fluids was analyzed with a Kruskal-Wallis one-way ANOVA and a Mann Whitney U posttest to compare each timepoint between treatment groups. Grubb's outlier test indicated one data point from corneal tissue data (Fig. 6A, 2 hr, OS cysteamine eyedrops) and was removed prior to statistical analysis. All statistical analyses were performed using Minitab software (State College, PA, USA).

3. Results

3.1. In vivo administration of SD-CMS/Gel

SD-CMS/Gel samples were freeze-dried, sectioned, gold sputtered and imaged with SEM to verify the morphology and presence of microspheres within gel prior to topical administration (Fig. 2A) and 24 hr after administration *in vivo* (Fig. 2B). The spray-dried microspheres aggregate prior to instillation *in vivo* which was anticipated from previous *in vitro* studies (Jimenez et al., 2021). After 24 hr, the microspheres appear to be more uniform. Photographs of the delivery systems in the rabbit model were acquired upon administration as seen in Fig. 2C. The delivery system was recovered and freeze dried (Fig. 2D) and use for SEM.

3.2. Instillation tolerability rabbit grimace and Draize eye tests

Four treatment groups: 0.9% saline, 0.44% cysteamine eyedrops, SD-BLANK-MS/Gel, and SD-CMS/Gel were administered to the right eye of subjects and their images recorded at specific time points (Fig. 3A). Images for each timepoint were scored by an ophthalmologist for rabbit grimace scores (RGS) at the following levels: 0 - discomfort not present, 1 - moderately present, 2 - obviously present. There were no statistically significant differences between the treatment groups at any given timepoint. Baseline scores (time = 0 min) for each treatment was captured to obtain a reference point for potential discomfort of the subject during anesthesia-free restraint. In addition to baseline scores, a negative control of saline provided a reference of any discomfort of administration of a well-tolerated aqueous solution. The saline group maintained an average RGS below 1 between 0.5 mins to 5 mins (Fig. 3B, RGS 0.333 ± 0.58). For the cysteamine eyedrop formulations, there was discomfort between 0.5 mins (Fig. 3B – cysteamine eyedrops, Fig. 3B – SD-CMS/Gel, Fig. 3B– SD-BLANK-MS/Gel) with average RGS 1–1.33 ± 0.58. After 10 mins, average rabbit grimace scores returned to respective baselines (time = 0 min), which indicates a trend of discomfort during instillation and a transient effect of administration. The images were also scored for Draize eye test, summarized in supplemental table 1 with scores for Cornea (0 – no ulceration or opacity) and Iris (0 – Normal). Scores for Chemosis and Conjunctiva where indicated had normal scores (0) for all groups.

3.3. Intraocular pressure monitoring

As expected, after administration of SD-CMS/Gel the average IOP of the treated eye (OD) did not vary significantly from that of the respective contralateral eye (OS). At any given timepoint, all eyes, whether treated or untreated were within normotensive ranges (15–23 mmHg) as presented in Fig. 4A-D.

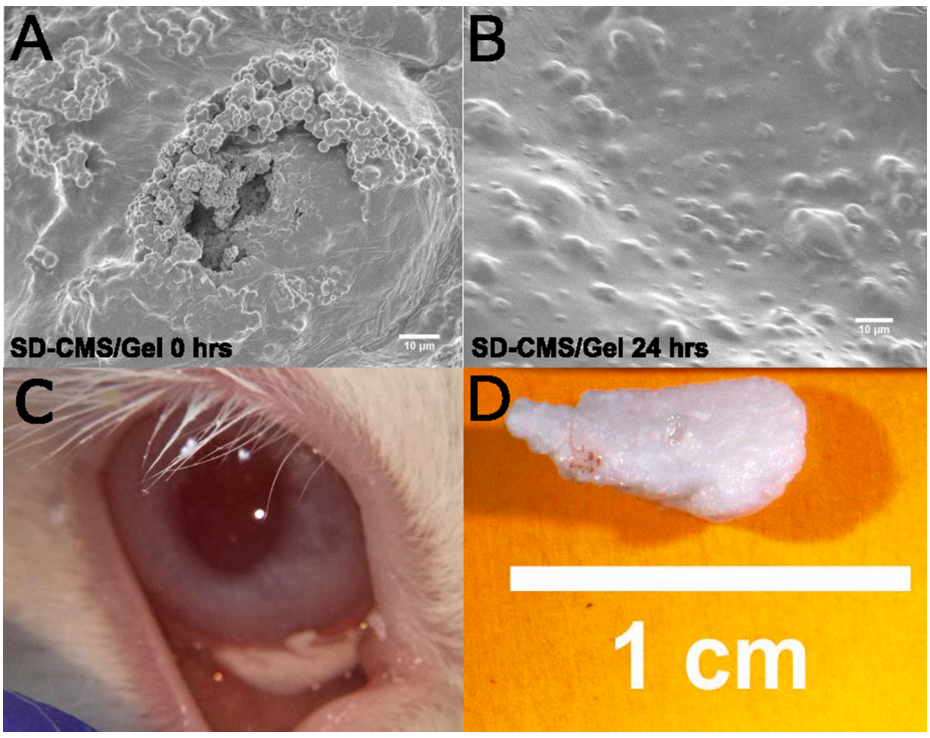

Fig. 2. Scanning electron microscopy images of SD-CMS/Gel prior to administration (A) and 24 h after *in vivo* administration (B), scale bar 10 µm. A representative photograph of SD-CMS/Gel placed in the lower fornix of the rabbit model (C) and the SD-CMS/Gel after 24 h, removed from eye, freeze-dried and imaged with a dissecting microscope, scale bar 1 cm (D).

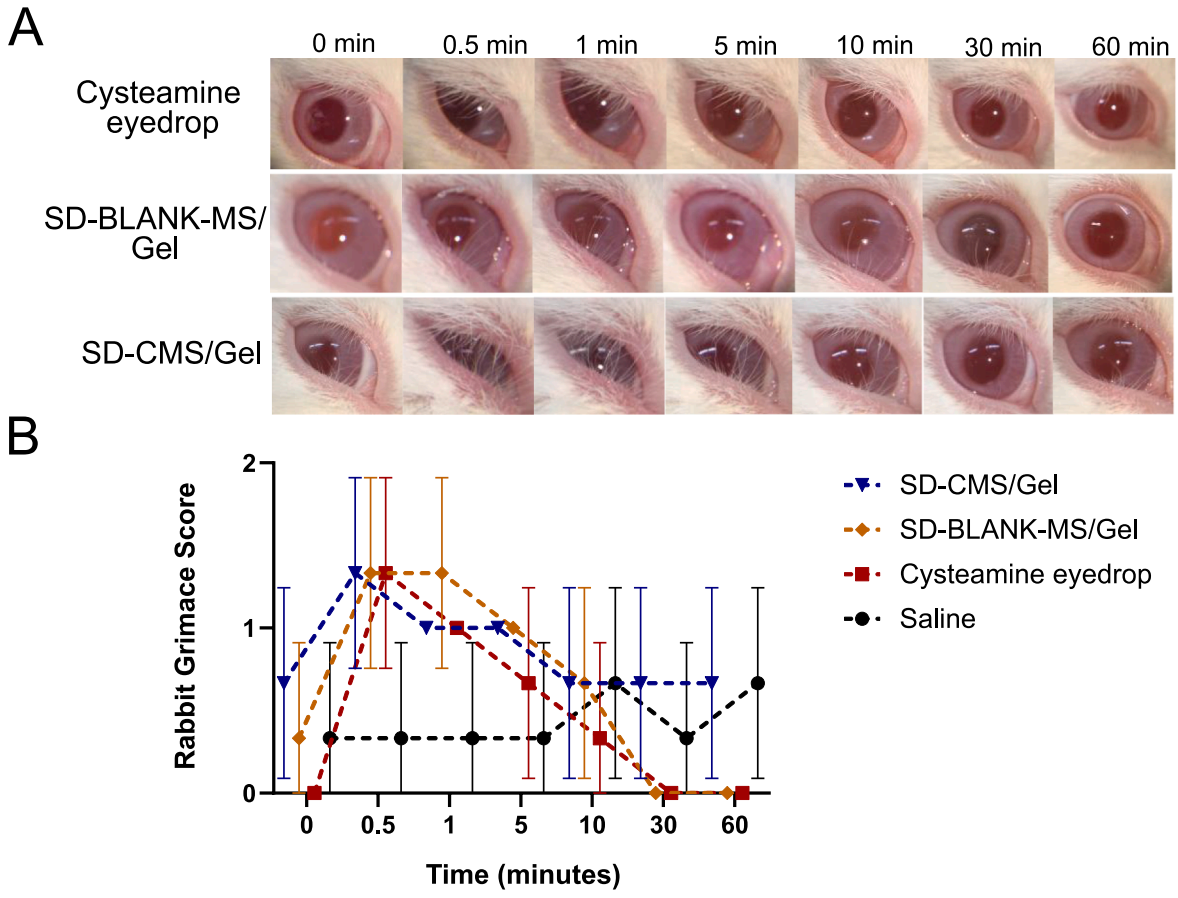

Fig. 3. Images acquired with hand held slit lamp for scoring instillation safety from eyedrop formulations and controls (A). Rabbit grimace score of eyedrops formulations and a 0.9% saline control (B). The data are represented as mean ± standard deviation for N = 3 subjects.

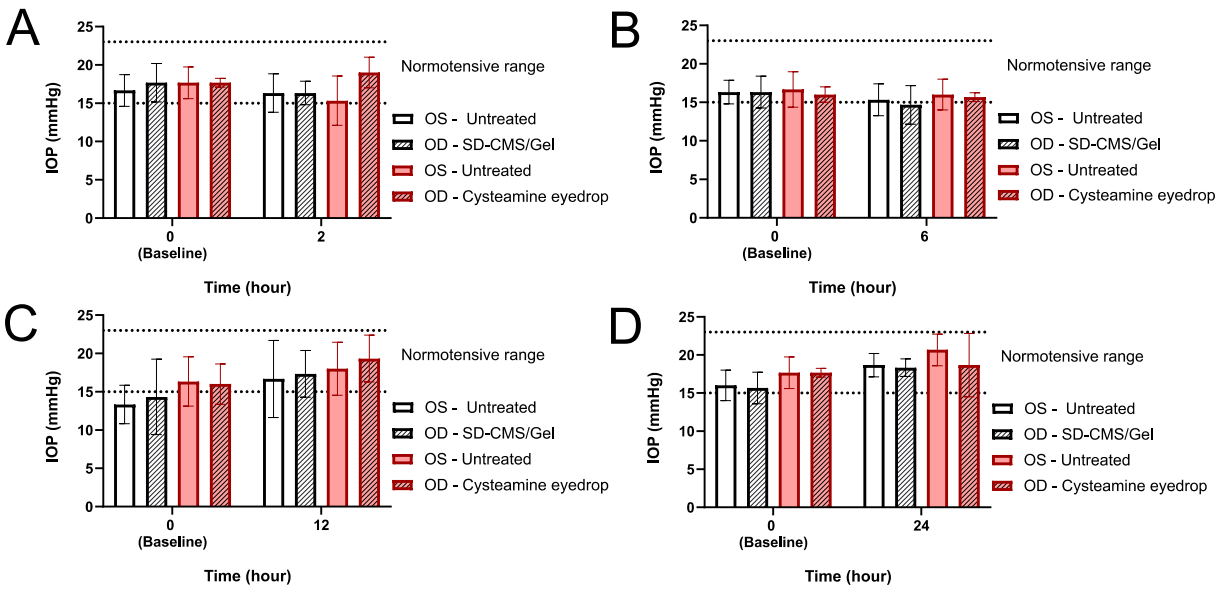

Fig. 4. Intraocular pressure (IOP) off treatment eyes (OD, SD-CMS/Gel, cysteamine eyedrop) and untreated contralateral eye (OS) at A.) 2 h, B.) 6 h, C.)12 h, and D.) 24 h. The data are represented as mean ± standard deviation for N = 3 subjects. Dotted lines represent normotensive range of IOP in New Zealand white rabbits (Lim et al 2005).

3.4. Histopathology of eyelids

Tissue samples from all timepoints (2, 6, 12, 24hr) were recovered and the 24 hr timepoint was selected to represent overall physiology and structural changes in Fig. 5. Microscopic images of eyelids from 2, 6, and 12 hr are within supplemental materials, Figures S.1-S.3. Untreated eyelid (Fig. 5A) H&E staining represent normal physiological and anatomical structures, such as an intact surface epithelium on the conjunctival side of the section – which is indicated by an asterisk (*). VVG staining of untreated eyes (Fig. 5B) suggest no effect on collagen or elastin (stained red). Cysteamine eyedrops (Fig. 5C-D) and SD-CMS/Gel (Fig. 5E-F) are comparable to untreated eyes.

3.5. Cysteamine distribution to ocular tissues and plasma

Cysteamine was topically delivered to the right eyes of rabbits at a frequency of administration of 12 hourly drops for cysteamine eyedrops and one drop of SD-CMS/Gel. For example, at 6 hr the cysteamine treated eye would receive 6 hourly drops and the SD-CMS/Gel would receive one drop at time 0 mins for the entire 6 hr time course. The contralateral eye was treated as independent for each timepoint. After serial sacrifice and tissue harvesting, cysteamine was extracted from corneas (Fig. 6A) and normalized to the weight of corneal tissue (e.g milligram). Cysteamine in fluid samples were also quantified in aqueous humor (Fig. 6B), vitreous humor (Fig. 6C) and plasma (Fig. 6D). Several samples were below the limit of detection (<15 nM) and were considered as a value of zero for plotting and statistical purposes. Statistical analysis indicated no statistical significance for ocular tissues and plasma mean values at any given timepoint. Based on descriptive statistics, cysteamine eyedrops presented a 3-fold magnitude higher cysteamine tissue concentration (pmol/mg) (e.g. 2 hr 66.93 ± 27.12, 6 hr 57.96 ± 25.45) than SD-CMS/Gel (2 hr 20.57 ± 11.15, 6 hr 29.48 ± 10.72). One data point in the 2 hr sample set was a statistical outlier and removed from the data analysis. After receiving 12 doses, cysteamine eyedrops at 12 hr was 5-fold higher than SD-CMS/Gel. Cysteamine was detected in the aqueous humor of treated eyes and follows a similar trend to corneal tissue – however, there was higher variability between samples in the cysteamine eyedrop treated eyes than SD-CMS/Gel treated eyes. Vitreous humor cysteamine concentrations were detected in all eyes, including the contralateral eye of subjects treated with cysteamine eyedrops and SD-CMS/Gel. Cysteamine was detected in plasma after 6 h of treatment (cysteamine eyedrops- 3.55 ± 40.79 nM, SD-CMS/Gel 8.82 ± 15.28 nM) and had cysteamine concentrations below the limit of detection after 24 hr.

4. Discussion

The instability of cysteamine eyedrops and frequency of administration add burden to cystinosis patients who must already manage multifaceted complications from their systemic disease with oral therapies and clinical care. The current cysteamine eyedrop formulations were approved based on efficacy studies where human participants received titrations of cysteamine concentrations at various dosing frequencies. The cornea and ocular tissues of patients were evaluated for corneal cystine crystal clearance, ultimately leading to a 0.55% cysteamine hydrochloride (HCL) eyedrop as an effective therapy in the EU (Iwata et al., August 1998; Kaiser-Kupfer, 1990; Bradbury et al., 1991; Tsilou et al., 2003). In the US these FDA-approved drops are labeled as 0.44% cysteamine HCL (Cystaran®) which is equivalent to 0.55% cysteamine HCL accounting for the moisture content of the HCL (Huynh et al., 2013; Makuloluwa and Shams, 2018) . Recently, the FDA-approval of a viscous formulation, Cystadrops®, contains 0.37% cysteamine HCl and prescribed at 4 times per day (Inc, Recordati Rare Diseases 2021), suggests that a lower cysteamine concentration with reduced frequency of administration may be obtained if the formulation resides on the ocular surface longer than aqueous solutions. Despite these advances, neither of the formulations were tested for ocular pharmacokinetics or biodistribution in large animals. To address the limitations of cysteamine stability, our group developed a topical formulation consisting of encapsulated cysteamine into PLGA microspheres and embedded within a thermoresponive gel for sustained release behavior *in vitro* for 24 hr (Jimenez et al., 2021). The sustained release formulation requires one drop to afford drug presentation at similar drug levels of prescribed cysteamine eyedrops. Data from our formulation resulted in 24 hr retention of the sustained release formulation in a New Zealand white rabbit model with low ocular irritancy. The present study further addresses the clinical need for cysteamine eyedrops with improved stability and low dosing frequencies by determining the ocular biodistribution of cysteamine after topical administration of cysteamine in eyedrops and a sustained release formulation.

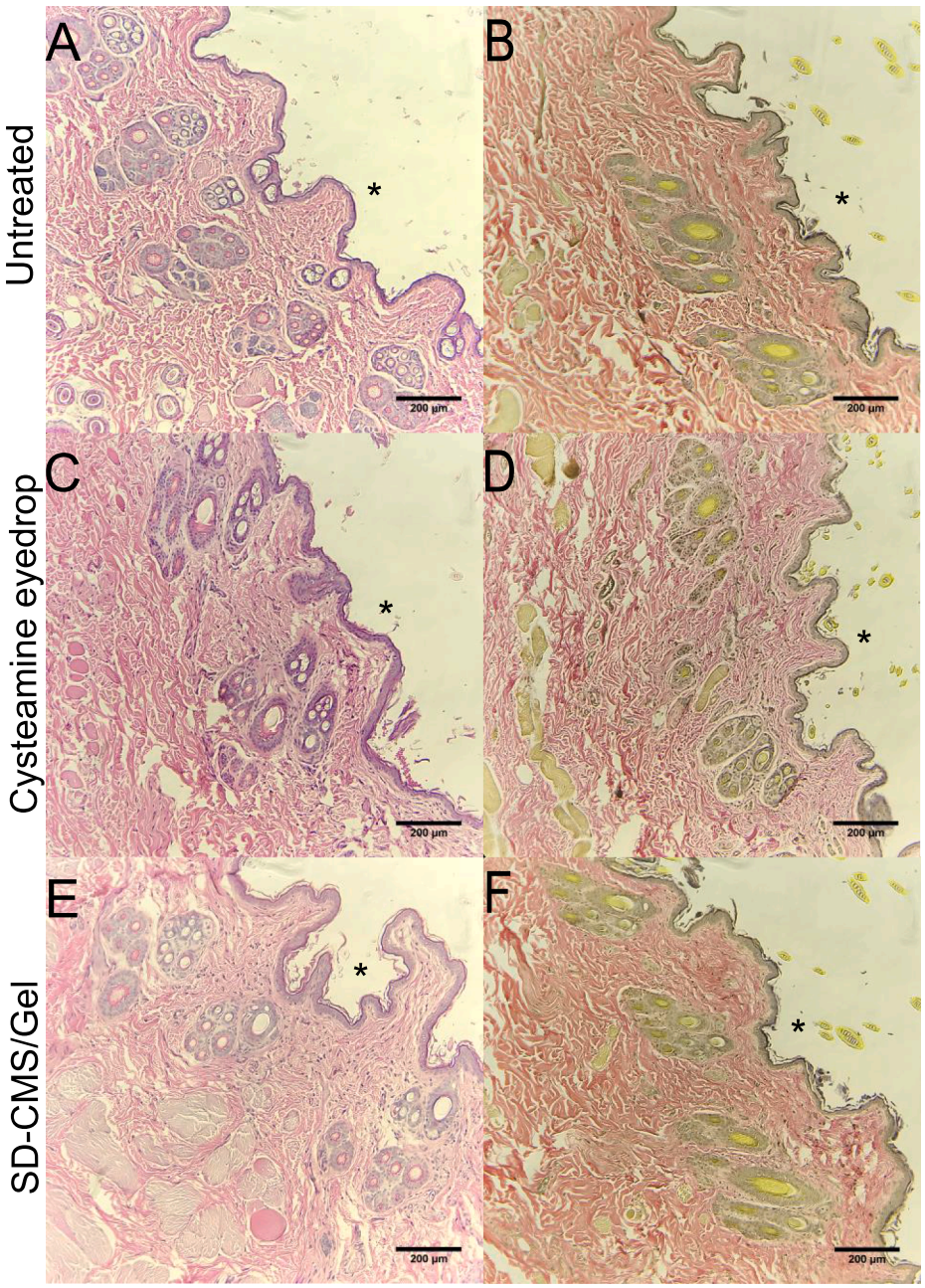

Fig. 5. Hematoxylin and eosin (H&E) and Verheoff Van Gieson (VVG) staining of eyelids of untreated eyes (A. H&E, B. VVG), cysteamine eyedrops (C. H&E, D. VVG), and SD-CMS/Gel (E. H&E, F. VVG). Histology captures treatment after 24 h. An asterisk (*) indicates the conjunctival side of the eyelid. Scale bar 200 μm.

To our knowledge, we present the first study quantifying cysteamine in ocular tissues and plasma after topical administration to the eye of rabbits. Current research on cysteamine controlled release technologies have only performed *in vivo* retention and biopermanance studies in rats (Luaces-Rodríguez et al., 2017) and efficacy without pharmacokinetic data in genetic cystinosis mice models (Marcano et al., 2016). Summary statistics from corneal tissue (Fig. 6A) indicate cysteamine delivered from cysteamine eyedrops at hourly doses delivered approximately 60 pmol/mg cysteamine/corneal tissue at 2 hr receiving 2 drops and 6 hr receiving 6 drops. One data point from this sample set (Fig. 6A, 2 hr, untreated cysteamine eyedrop OS), was a statistical outlier with a drug concentration beyond treated levels (1360 pmole/mg v.s. 60 pmol/mg). The SD-CMS/Gel achieved approximately 20 pmol/mg cysteamine/corneal tissue from one drop at the same time points. A full daily course of multiple doses (12 drops) of cysteamine eyedrops reached 5 times as much drug than SD-CMS/Gel at 12 hr. Although there are no comparable corneal tissue data, a pharmacokinetic study on rats after catheter intraduodenal delivery of 20 mg/kg cysteamine achieved cysteamine liver concentrations of 0.2 nmol/mg protein at 6 h and 0.11 nmol/mg protein at 24 hr (Dohil et al., 2014). These values are not directly comparable because the delivery methods are drastically different and the cysteamine tissue concentration in this study was normalized to protein content, however, it can be estimated that cysteamine presentation during topical application is on a scale of magnitude a thousand times less than intraduodenal delivery, where topical delivery achieved nanomolar cysteamine concentrations and intraduodenal delivery achieved micromolar cysteamine concentrations.

Additional ocular samples consisting of aqueous humor and vitreous humor were quantified for cysteamine concentrations. Aqueous humor samples in the treated eyes achieved drug presentation at all timepoints with cysteamine eyedrops having wider variation in samples compared

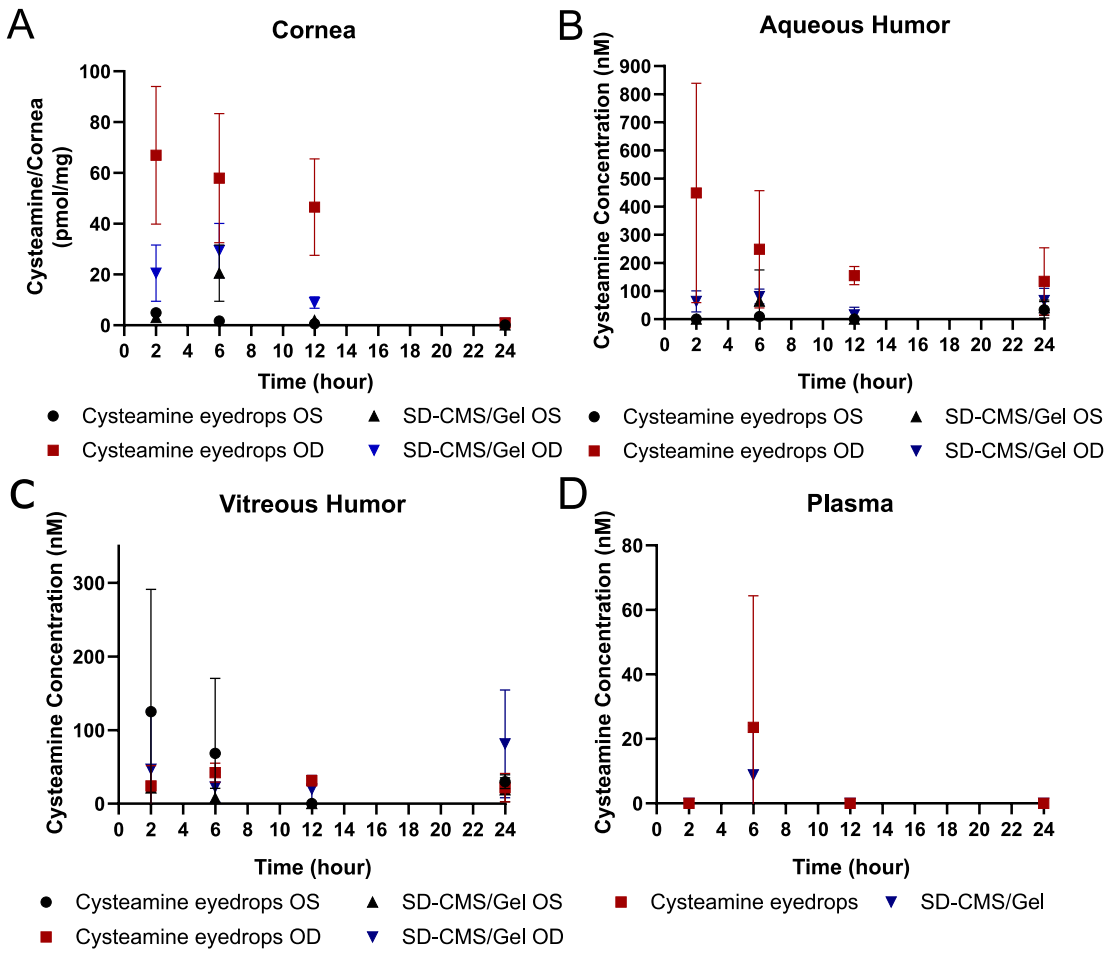

Fig. 6. Cysteamine quantified after topical administration of cysteamine eyedrops and SD-CMS/Gel in A.) corneal tissue and normalized based on tissue weight (pmol/mg), B.) aqueous humor concentration (nM), C.) vitreous humor concentration (nM), and D.) plasma. Ocular tissue samples include the contralateral, untreated left eye (OS) and treated right eye (OD) and are categorized by respective eyedrop formulations. The data are represented as mean ± standard deviation for N = 3 per timepoint. In A). cysteamine eyedrops, OS is reported as N = 2 with one data point considered a statistical outlier.

to SD-CMS/Gel. For example, at 6 hr cysteamine eyedrops delivered 248.7 ± 208.55 nM compared to SD-CMS/Gel 80.67 ± 26.47 nM. Interestingly, the untreated contralateral eyes at 2 hr had no detectable cysteamine concentrations (<15 nM) in all samples while some samples had detectable drug amounts with wide variation at subsequent timepoints for both formulations (Fig. 6B and Fig. 6C). Potential crosstalk between contralateral eyes may explain this phenomenon and is further speculated in our analysis of vitreous humor samples. Several samples from untreated eyes presented cysteamine at detectable concentrations. These concentrations were less than aqueous humor levels at the same magnitude, which may indicate less posterior segment drug adsorption as reported in other pharmacokinetic rabbit studies of small molecules delivered topically (Lin et al., 2015).

Our study also revealed a trend of cysteamine plasma concentrations below the lower limit of quantitation at 2, 12, and 24 h (Fig. 6D). At 6 h, there was detectable cysteamine concentrations in the plasma from subjects treated with cysteamine eyedrops and SD-CMS/Gel near the lower limit of detection. For all other time points, there were no detectable cysteamine concentrations. These observations are particularly important due to the inability to obtain peak plasma concentration of cysteamine following ocular administration of cysteamine during clinical trials in humans. It is likely there is low plasma concentrations of cysteamine during a 24 h period when cysteamine is topically delivered. Patients enrolled in these trials were pretreated with prescribed oral cysteamine which is far greater than one daily ophthalmic dose of cysteamine eyedrops (Makuloluwa and Shams, 2018) and would be the main contributor of plasma cysteamine concentrations and not eyedrops. We furthered evaluated the translation of the sustained release formulation with instillation tolerability studies and semi-quantitative clinical scoring.

We evaluated rabbit grimace pain scales and Draize eye test for irritation during eyedrop instillation between cysteamine eyedrops, our delivery system materials without cysteamine (SD-BLANK-MS) and cysteamine-loaded materials (SD-CMS/Gel). These studies were guided by adverse effects observed in clinical trials as noted by redness expanding over 50% of the conjunctiva (Tsilou et al., 2003) and transient effects lasting<1 h which included stinging and burning (Labbé et al., 2014; Liang et al., 2017). Based on our model and clinical scoring by an ophthalmologist, the formulations were well tolerated and any pain upon instillation was relieved between 10 and 60 mins (Fig. 3A&B). This was expected as the clinical trial observers saw relief after 1 hr. Clinical trial investigators also suspected that any increase in resident time of a formulation as well as a higher concentration of cysteamine HCL may cause discomfort from viscous cysteamine studies (Liang et al., 2017; Liang et al., 2015). Draize eye test scores also supported transient effects of irritation lasting up to 10–30 mins as noted in Supplemental table 1. During these instillation tolerability studies, the non-degradable depot was easily removed from the lower eyelid space by gently pushing the depot upward from outside the eyelid. The gel material can also be extracted with a cold saline flush as reported in previous glaucoma studies (Fedorchak et al., 2017). When considering translation to pediatric patients with cystinosis, a guardian would ideally administer and

remove the depot until patients can perform self-administration. Overall, the sustained release materials were tolerated during instillation in the pre-corneal area. t. In addition to instillation tolerability, we also monitored the safety profile of subjects prior to tissue collection for intraocular pressure and structural changes to eyelids after treatment with histopathology.

The intraocular pressure of rabbits in untreated and treated eyes was quantified. Overall, all measurements were within normotensive range 15–23 mmHg (Lim et al., 2005; Vareilles et al., 1977) for all subjects at each timepoint (Fig. 4A-D). There was no increase in IOP from any of the formulations, which was expected and supported by previous rabbit studies conducted by our group (Fedorchak et al., 2017). Histopathological analysis of eyelid tissue was used as a proxy for irritation at the ocular surface since the inner surface of the eyelid (palpebral conjunctiva) is similar to the scleral surface of the eye (bulbar conjunctiva) and respond to similar irritant effects (York and Steiling, 1998). H&E staining revealed no structural differences in untreated and treated eyes with an intact conjunctival epithelium in all samples at 24 hr (Fig. 5A-F). VVG staining also presented no changes in elastin or collagen as indicated by uniform red structures observed in all samples. Thus, the visible similarities between sections suggest no effect of cysteamine and materials in SD-CMS/Gel effect the structure of eyelids. We previously performed irritation assays on hen's eggs and bovine eyes which observed little to no irritation within 8 hr (Jimenez et al., 2021) and our eyelid histology agrees with these findings. It is important to note that we were unable to obtain corneal histology due to the tissue processing for cysteamine extraction with subsequent mass spectrometry analysis.

In addition to our limitation in obtaining corneal histology, the challenges to maintaining cysteamine stability during *in vivo* topical administration and tissue post processing may contribute to variability observed in our mass spectrometry data. At administration, cysteamine may be exposed to oxidative degradation while simultaneously metabolized endogenously into thiol derivatives (e.g S-methylcysteamine and hypotaurine) (Atallah et al., 2020). The tissue sample processing with NEM for mass spectrometry targets the free sulfhydryl group in cysteamine; if the sulfhydryl group is blocked, as in the disulfide bond formation in cystamine, then cysteamine cannot be quantified with NEM derivatization. Therefore, the data presented is a best-case scenario for quantifying active cysteamine after quickly and humanely excising tissue post-mortem. To overcome this, adding a reducing agent such as tris (2-carboxyethyl)phosphine prior to NEM derivatization would reduce cysteamine thiol derivatives to determine total cysteamine prior to oxidation. Subsequent mass spectrometry analysis would thus require validation to ensure extraction ratios are not inhibited by the reducing agent. Furthermore, the variability in our biodistribution data may be lessened by increasing the current sample size (N = 3) for each time point to a sample size large enough for statistical power with careful consideration of retaining the ability to quickly sparse sample subjects. The literature supports the use of satellite groups (Tuntland et al., 2014), which are subjects undergoing pharmacokinetic studies only, for sparse sampling performed in the current studies across four timepoint. A future *in vivo* study consisting of earlier timepoints (1 min, 5 min, 10 min, 30 min, 60 min) with a single dose of cysteamine eyedrops may provide a pharmacokinetic profile of cysteamine when topically delivered. We did not investigate the pharmacokinetic profile of a single drop of cysteamine eyedrops. Rather, our study replicated the prescribed dosing regimen of Cystaran® eyedrops and readministered every hour up to the 12th hour. In doing so, we developed a biodistribution model that is in direct translation to patients who administer cysteamine eyedrops at every waking hour and provides a basis for comparing future ophthalmic formulations. Lastly, it is critical to determine efficacy of the sustained release formulation by reducing cystine crystals in the cystinosis mouse (Cherqui et al., 2002) or a rat model, which is currently being developed (Hollywood and Kallingappa, 2021).

5. Conclusion:

An *in vivo* ophthalmic model consisting of the New Zealand white rabbit was utilized to provide ocular tissue and systemic cysteamine concentrations after topical administration of our sustained release gel formulation and traditional eyedrops. Multiple doses of aqueous cysteamine eyedrops, when administered hourly, maintained drug concentrations within the cornea at a magnitude 5 times higher than a single dose of our technology over 12 h. Despite the difference in drug uptake, the sustained release formulation maintained drug release across 12 h from a single drop, potentially reducing the need to readminister by 8–11 drops. Systemic uptake of cysteamine from our formulation was below our limit of detection after plasma cysteamine concentrations were quantified during sparse blood sampling. During these studies, clinical scores from an ophthalmologist indicated our sustained release formulation and controlled release materials without drug were tolerable and any observed transient effects were diminished within 10–30 mins. Histological evaluation of eyelid tissue served as a proxy for irritation at the ocular surface during *in vivo* studies and observed no structural changes. These histopathological findings were comparable to the effect from traditional cysteamine eyedrops. In total, these studies inform the first large animal ocular biodistribution of multiple doses of cysteamine eyedrops – when previous studies failed to provide imperative ocular tissue drug levels.

CRediT authorship contribution statement

Jorge Jimenez: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing – original draft. **Jayde L. Resnick:** Investigation. **Ahmad B. Chaudhry:** Investigation. **Ilya Gertsman:** Methodology, Validation. **Ken K. Nischal:** Conceptualization, Formal analysis. **Morgan V. DiLeo:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Morgan V. DiLeo reports financial support was provided by Cystinosis Research Foundation. Jorge Jimenez reports financial support was provided by National Institutes of Health. Morgan V. DiLeo reports financial support was provided by National Institutes of Health. Morgan V. DiLeo reports financial support was provided by Research to Prevent Blindness. Morgan V. DiLeo has patent issued to University of Pittsburgh.].

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2022.121992.

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