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Silvia Pescina, Federica Carra, Cristina Padula, Patrizia Santi, Sara Nicoli*

Department of Pharmacy, University of Parma

*Corresponding author
Sara Nicoli PhD
Department of Pharmacy
University of Parma
Parco Area delle Scienze, 27/A
43124 Parma, Italy
Telefono +39 0521 905065/71
Fax +39 0521 905006
E-mail: sara.nicoli@unipr.it

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ABSTRACT

Ocular cystinosis is a rare metabolic disorder characterized by the presence of insoluble cystine crystals inside the corneal stroma, with consequent photophobia, keratopathies and frequent corneal erosions. The current therapy consists in the lifetime ophthalmic administration of cysteamine, drug characterised by extremely high hydrophilicity, low molecular weight (77 g/mol), and easily oxidized to disulphide. Ocular delivery of cysteamine is very challenging, for its poor permeability and stability in solution. The purpose of the present paper was to study the impact of formulation pH and composition on 1) the trans-corneal delivery and 2) the stability in solution of cysteamine, with particular focus on the use of alpha-cyclodextrin (α-CD), benzalkonium chloride (BAC) and disodium edetate (EDTA). Permeation experiments were performed *ex vivo* through freshly excised porcine cornea; stability was evaluated for six months at -20°, +4° and +25°C; irritation potential was evaluated using HET-CAM assay. The results showed that cysteamine trans-corneal diffusion is strictly dependent on both pH (7.4 preferred to 4.2) and buffering capacity, that negatively impact on the permeation; EDTA did not enhance the trans-corneal diffusion of cysteamine neither at pH 7.4 nor at pH 4.2, while benzalkonium chloride (BAC), antimicrobial agent present within commercial eye-drops, significantly enhanced it. Notably, α-CD was able to promote the trans-corneal diffusion of cysteamine and, at a 5.5%, a 4-fold higher penetration compared to the BAC-containing formulation was obtained. EDTA and acidic pH demonstrated to be essential for cysteamine stability. The formulation obtained by combining α-CD and EDTA was characterized by significant permeation, good stability profile, and no irritation potential, even if the tolerability should be further confirmed by *in vivo* test.

1 INTRODUCTION

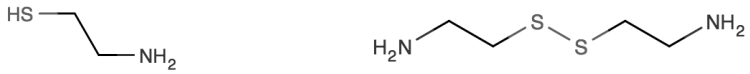
Cysteamine is an orphan drug [1, 2] successfully used for treating cystinosis, a rare metabolic disorder caused by the mutation of CTNS gene that encodes for the lysosomal protein cystinosin. The diseases causes the accumulation of insoluble cystine crystals (i.e. the disulfide of the amino-acid cysteine) inside the cells of several organs and tissues, particularly within kidney and eye [3]. Oral administration favorably treat systemic symptoms [4], but is completely ineffective for ocular manifestations since the cornea, as a consequence of the angiogenic privilege, is an avascular tissue. Therefore, the only possible approach for the effective decrease of corneal cystine crystals responsible for photophobia, keratopathies and frequent corneal erosions [5], is the topical administration of the drug. FDA has recently approved Cystaran™, a cysteamine hydrochloride based eye-drop effective in the reduction of corneal cystine crystals [6].

Ophthalmic delivery of cysteamine is very challenging, for its poor permeability and stability in solution. The diffusion of the highly hydrophilic molecule across the lipophilic corneal epithelium is highly hindered, thus constraining to hourly administration of eye-drops during daytime. Moreover, in aqueous solution, cysteamine tends to oxidize to cystamine (Figure 1), a non toxic, but ineffective compound [7]. For these reasons the eye-drop Cystaran™ has to be administered hourly during daytime; the product has to be stored at -20°C and, after opening, it should be used within 1 week, keeping it between +2°C and +25°C [8].

In order to improve cysteamine ocular administration, some formulative approaches have been recently proposed. Among others, the increase of residence time of the solution on the ocular surface by means of thickening agents i.e. hydroxypropylmethylcellulose [9] and carbomer [10]; the control of release of the drug using sodium carboxymethyl cellulose hydrogels [11] [12] and Poloxamer 407 [12] or silicone contact lenses containing vitamin E [13]; nanowafer [14]. Additionally, a few papers address the problem of cysteamine stability by adding EDTA [15] or using reconstructed gels [16]. Despite the formulative efforts, no attention has been dedicated until now to elucidate the impact of the formulation on corneal delivery and, surprisingly, no information is present in the literature, to our knowledge, on cysteamine trans-corneal transport.

The purpose of the present paper was to study the impact of formulation on cysteamine trans-corneal permeation and stability. The role of pH, EDTA and benzalkonium chloride (BAC) was at first evaluated. BAC is an antimicrobial agent present in the commercial formulation that may cause eye irritation [17] but, at the same time, can have an important

activity as permeation enhancer [18]. Then, the use of α-CD as excipient was investigated. The final aim was both to increase drug delivery to the cornea– thus reducing the number of administrations per day - and to increase cysteamine stability in aqueous solution.



Cysteamine Cystamine

Figure 1. Molecular structure of cysteamine and cystamine.

2 MATERIALS AND METHODS

2.1 Materials

Cysteamine as hydrochloride (MW 113.61 g/mol, MW as free base 77.15 g/mol; pK_{a1} 8.32, pK_{a2} 10.34; $LogD_{7.4}$ -2.36 [19]) as well as cystamine as dihydrochloride (MW 225.20 g/mol, MW as free base 152.28 g/mol), were a gift from Recordati (Milan, Italy); disodium edetate (EDTA) was from Alfa Aesar (Karlsruhe, Germany); benzalkonium chloride (BAC) was purchased from Sigma Aldrich (Sigma-Aldrich, St. Louis, USA). alpha-cyclodextrin (α-CD; MW 972.84 g/mol) was from Wacker Chemie (Munich, Germany); any other materials, including solvents for liquid chromatography, were of analytical grade. Cysteamine hydrochloride solutions (0.44% w/v as free base) were obtained by dissolving the drug in the vehicles listed in Table 1. The solutions pH was then adjusted to the desired value using 0.1 M HCl or 0.1 M NaOH.

2.1.1 Permeation experiments

Trans-corneal diffusion was studied using an *ex vivo* model consisting of isolated freshly-excised porcine corneas, as previously described [20]. Briefly, full-thickness (epithelium, stroma and endothelium) or de-epithelialized (stroma and endothelium) corneas from pigs (Large White and Landrace, 145-190 kg, 10-11 months) were isolated and mounted on Franz-type diffusion cells, having a permeation area of 0.2 cm^2, within 3 hours from animal death. The donor compartment was filled with 0.3 ml of 0.44% cysteamine solution in different vehicles (Table 1), while pH 7.4 HEPES buffer added with 0.01% EDTA was used to fill the receiving compartment (*approx*. 4 ml volume).

Table 1 Composition of cysteamine hydrochloride solutions (0.44% w/v as free base).

#	NaCl (%)	BAC (%)	EDTA (%)	HEPES (%)	NaH_2PO_4 (%)	alpha CD (%)	pH
C_0	0.9	0.01	-	-	-	-	4.2
C_1	-	0.01	0.1	-	1.85	-	4.2
C_2	0.9	-	0.01	0.65	-	-	7.4
C_{3a}	0.9	-	-	-	-	-	4.2- 4.4
C_{3b}	0.9	-	-	-	-	-	7.4
C_{4a}	0.9	-	0.1	-	-	-	4.2
C_{4b}	0.9	-	0.1	-	-	-	7.4
C_5	0.9	0.01	-	-	-	0.06	4.2
C_6	0.9	0.01	-	-	-	1.5	4.2
C_{7a}	0.9	0.01	-	-	-	3	4.2
C_{7b}	0.9					3	4.2
C_{8a}	0.9	0.01	-	-	-	4.25	4.2
C_{8b}	0.9	-	-	-	-	4.25	4.2
C_{9a}	0.9	0.01	-	-	-	4.9	4.2
C_{9b}	0.9	-	-	-	-	4.9	4.2
C_{9c}	0.9	-	0.1	-	-	4.9	4.2
C_{9d}	0.9	-	0.1	-	1.85	4.9	4.2
C_{10a}	0.9	0.01	-	-	-	5.5	4.3
C_{10b}	0.9	-	-	-	-	5.5	4.3

Preliminary studies demonstrated that the presence of 0.01% EDTA prevented cysteamine oxidation for at least 24 hours. Receptor phase, previously degassed, was continuously stirred and kept in a 37°C water bath throughout the experiment time. In order to quantify cysteamine permeated, 0.3 ml of receptor solution were collected at predetermined times (0, 20, 40, 60, 80, 100, 120, 180, 240, 300 minutes) over a 5 hours period and analysed by HPLC-UV. Each condition was replicated at least 3 times and, in order to reveal possible interferences from the tissue, blank assays were conducted. pH values of donor solution were monitored over time by means of a microelectrode.

Data are presented as amount of cysteamine permeated (μg/cm^2) as a function of time (min). The trans-corneal flux of cysteamine through both full-thickness and de-epithelialized corneas (J, μg/cm^2 min) was calculated as the slope of the regression line at steady state, while the apparent permeability coefficient of full-thickness (full) and de-epithelialized (de-epi) cornea (P_{app}, cm/s) were calculated at the steady state as:

$P_{app} = J/C_D$ **equation 1**

were C_D (µg/ml) is the concentration of the donor solution.

The apparent permeability coefficient of the epithelium was calculated using the resistance approach [21]. The resistance to transport (R) can be written as:

$R_{cornea} = R_{epithelium} + R_{stroma} + R_{endothelium}$ **equation 2**

Since the resistance (R) is the inverse of permeability (P_{app})

$$\frac{1}{P_{cornea}} = \frac{1}{P_{epithelium}} + \frac{1}{P_{stroma}} + \frac{1}{P_{endothelium}} \qquad \textbf{equation 3}$$

that can be written as:

$$\frac{1}{P_{full}} = \frac{1}{P_{epithelium}} + \frac{1}{P_{de\text{-}epi}} \qquad \textbf{equation 4}$$

Enhancement Factor (EF) was calculated by comparing obtained permeability coefficients (P_{test}) to the permeability coefficient of the reference formulation C_0 (P_{C0}):

$$EF = \frac{P_{test}}{P_{C_0}} \qquad \textbf{equation 5}$$

Attempts were done to validate an extraction procedure in order to be able to quantify the amount of cysteamine accumulated inside the cornea, however the recovery % was always very low. Preliminary experiments were performed to evaluate cysteamine stability in contact with the corneal tissues: 9 mm diameter full-thickness cornea punches were soaked in cysteamine solutions (pH 7.4, 0.01% EDTA) at different concentration and analysed by HPLC after up to 5 of contact at room temperature.

2.1.2 HET-CAM assay

Hen's Egg Test Chorioallantoic Membrane (Het-Cam Assay) was performed as reported in ICCVAM-recommend test method protocol [22].

Fertilized chicken eggs (White Leghorn) at 9th day of incubation, having a weight of *approx.* 60 g, were purchased from Istituto Zooprofilattico della Lombardia e dell'Emilia-Romagna (Brescia, Italy). After cleaning the eggs with 70% ethanol, the eggshell was circumferentially cut with a surgical blade at the air cell. Once the inner membrane has been exposed, it was moistened using *approx.* 1 ml of saline solution (0.9% NaCl) and then carefully removed by tweezers. 100 µl of control or test solutions were added onto the cam and the observation was performed at 0.5, 2 and 5 minutes, recording any lysis, hemorrhage or coagulation

occurred, and assigning the irritation score (IS). IS is a value that, in accordance with ICCVAM-recommended protocols [22], is calculated by adding the score of each observation and can vary between 0 (no alterations are detectable) and 21 (lytic, hemorrhagic and coagulative phenomena observed). Four different samples were tested: solutions containing 5.5% α-CD with or without 0.44% cysteamine (C_{10b}) and solutions prepared by mixing 5.5% α-CD and 0.01% BAC with or without 0.44% cysteamine (C_{10a}). 0.9% NaCl solution was used as negative control. Each condition was tested in triplicate.

2.1.3 Dynamic Light Scattering

Vehicles prepared by dissolving 4.9% α-CD in distilled water and in the vehicle C_{9d} with or without 0.44% cysteamine were analysed by Dynamic Light Scattering (DLS) using a 90Plus/BI-MAS apparatus (Brookhaven Instruments Corporation, Holtsville, NY, USA). Both solution were filtered across 0.2 μm cutoff regenerated cellulose membrane (Sartorius AG, Goettingen, Germany). Each measure, repeated 6 times, lasted 1 min and was performed at 25°C, 658 nm laser beam, 90° scattering angle (fluid refractive index: 1.33, viscosity 0.89 cP).

2.1.4 Stability studies

Stability studies were performed on vehicles C_0, C_1, C_2, C_{7a}, C_{9b}, C_{9c} and C_{9d} (Table 1) over a 24 weeks period. Aliquots of 0.4 ml were transferred into polypropylene or glass tubes and stored at -20°C, 4°C and +25°C, protected from light. Cysteamine concentration was assessed by HPLC-UV after dilution with water. Before the chromatographic analysis, pH values were measured with a microelectrode. Each condition was tested in triplicate. In the case of C_0, C_1, C_2 the oxidation product cystamine was quantified as well.

2.1.5 Analysis

HPLC analysis was carried out using a system consisting of an isocratic pump (series 200 Perkin Elmer, Walthman, MA, USA), an autosampler (Prostar 410, Varian, Leinì, Italy) and a UV-Vis detector (SPD-20ALC Shimadzu, Kyoto, Japan), managed by Turbochrom work station software (Perkin Elmer). In alternative, an Agilent 1260 Infinity system (Agilent Technologies, Santa Clara, CA, USA) was used.

Two different methods were set up. For stability studies, cysteamine and cystamine were quantified simultaneously using an Alltima C18 LL 5 μm 250X4.6 mm column (Grace,

Columbia, MD, USA) thermostated at 50°C. Mobile phase consisted of a mixture of 380 ml water, 300 ml CH_3CN, 320 ml CH_3OH, added with 1.4 ml H_3PO_4 85% and 11.52 g sodium dodecyl sulphate pumped at 1.6 ml/min. Absorbance was recorded at 210 nm. Retention times were *approx.* 4 min and 14 min for cysteamine and cystamine, respectively. Linearity was between 50 µg/ml and 2000 µg/ml for both compounds.

Samples from permeation experiments (only cysteamine was quantified) were analyzed with a Jupiter C18 5 µm column (300 Å, 150X4.6 mm; Phenomenex, LePeq, France) thermostated at 50°C. Mobile phase was prepared by adding 1.4 ml of H_3PO_4 85% and 11.52 g of sodium dodecyl sulphate to a mixture composed of water (620 ml), CH_3CN (330 ml) and CH_3OH (50 ml) pumped at 1.6 ml/min. Absorbance was detected at 210 nm. Retention time was 4 min and linearity was between 5 µg/ml and 50 µg/ml. The LOD (limit of detection) was 1 µg/ml. Possible interference due to the cornea were evaluated. Standard solutions were prepared in HEPES pH 7.4 containing 0.01% EDTA; RSD% (relative standard deviation %) and ER% (relative error %) resulted lower than 5% and 10% respectively for all the concentrations tested.

3 RESULTS AND DISCUSSION

Cysteamine ocular administration faces three major challenges: 1) partitioning into and diffusion across the corneal epithelium, because cysteamine is a charged hydrophilic compound; 2) short residence time on ocular surface that, together with the low permeability, implies the need for frequent administrations; 3) instability of cysteamine which, in aqueous solution, tends to oxidize to cystamine (Figure 1), a non toxic, but ineffective compound.

Formulative approaches proposed until now were mainly focused to increase cysteamine retention on the ocular surface and/or control drug release, so as to reduce the administration frequency [9] [10] [11] [12] [13] [15] [14]. As a result, a CMC gel has completed a phase III study in Europe [23].

The aim of the present paper was to increase both the accumulation in the corneal stroma (here measured as trans-corneal permeation) and the stability of cysteamine in solution.

3.1 Trans-corneal permeation of cysteamine

The target of ocular cysteamine is the corneal stroma, where most of cysteine crystals are located [24]; for this reason it would be interesting to measure the drug level inside the tissue. An attempt to set up and validate an extraction procedure of cysteamine from the

cornea was done, by spiking the tissue with a known amount of the drug and extracting it in different conditions. Unfortunately, this method was unsuccessful: in fact, preliminary experiments demonstrated that cysteamine levels in a solution in contact with the cornea rapidly declined. This was probably the result of oxidative reactions, both enzymatic and non-enzymatic, commonly occurring to free thiol groups in mammalian cells [25] and therefore reasonably also inside freshly-excised cornea. Conversion of cysteamine to hypotaurine, taurine [26] and glutathione [27] has been reported. In addition, interactions between cysteamine and cornea components cannot be excluded. The difficulty in the quantification of cysteamine inside the cornea is witnessed by the absence of data in the literature.

In order to evaluate the performance of the different formulations prepared, the trans-corneal permeation through freshly excised porcine cornea was measured, assuming a direct correlation between the amount of cysteamine permeated across the cornea and the amount retained in the corneal stroma, *i.e.* the main target site. This assumption is reasonable considering that the permeation barrier is located in the epithelium. The permeability coefficients obtained using different vehicles, whose composition is detailed in Table 1, are reported in Table 2; permeation profiles are shown in Figures 2 and 3. The composition of formulation C_0 is the same as the commercial eye-drop.

A general consideration can be made concerning the lag time that is considerably long: regardless the formulation, cysteamine is never found in the receptor compartment before 60/80 minutes (Figure 2 and 3). This can be attributed to both the low permeability of this drug, and to its retention/metabolism inside the cornea, as previously detailed.

Table 2. Permeability coefficients of cysteamine through porcine cornea (mean values ± sd)

vehicle	*composition*†	P_{full} ± sd (n) $*10^{-6}$ a	$P_{de\text{-}epi}$ ± sd (n) $*10^{-6}$ a	$P_{epithelium}$ $*10^{-6}$ b	*EF* c
C_0	NaCl-BAC-4.2	7.41 ± 2.71 (7)	24.10 ± 3.20 (3)	9.59 ± 0.55	-
C_1	BAC-EDTA-phosp-4.2	5.68 ± 1.06 (6)	18.81 ± 4.19 (3)	7.95 ± 0.75*	0.8
C_2	NaCl-EDTA0.01-hepes-7.4	3.08 ± 1.54 (3)	19.82 ± 1.71 (4)	3.05 ± 0.04	0.4
C_{3a}	NaCl-4.2	n.c.(4)	n.d.	-	n.c.
C_{3b}	NaCl-7.4	2.60 ± 0.75 (4)	n.d.	-	0.4
C_{4a}	NaCl-EDTA-4.2	n.c. (4)	n.d.	-	n.c.
C_{4b}	NaCl-EDTA-7.4	2.97 ± 0.78 (4)	n.d.	-	0.4
C_5	NaCl-BAC-**α0.06**-4.2	4.02 ± 0.89 (3)	n.d.	-	0.5
C_6	NaCl-BAC-**α1.5**-4.2	0.47 ± 0.81 (3)	n.d.	-	0.1

C_{7a}	*NaCl-BAC-***α3**-4.2	3.95 ± 0.39 (3)	n.d.	-	0.5
C_{7b}	*NaCl-***α3**-4.2	6.92 ± 2.38(3)	n.d.	-	0.9
C_{8a}	*NaCl-BAC-***α4.25**-4.2	10.33 ± 1.94 (3)	n.d.	-	***1.4***
C_{8b}**	*NaCl-***α4.25**-4.2	14.89 ± 1.33 (5)	n.d.	-	***2.0***
C_{9a}**	*NaCl-BAC-***α4.9**-4.2	17.19 ± 2.57 (3)	n.d.	-	***2.3***
C_{9b}**	*NaCl-***α4.9**-4.2	19.05 ± 2.88 (5)	n.d.	-	***2.6***
C_{9c}**	*NaCl-EDTA-***α4.9**-4.2	21.97 ± 0.76 (4)	n.d.	-	***3.0***
C_{9d}**	*NaCl-EDTA-phosp-***α4.9**-4.2	21.39 ± 3.17 (5)	n.d.	-	***2.9***
C_{10a}**	*NaCl-BAC-***α5.5**-4.3	32.05 ± 6.42 (5)	n.d.	-	***4.3***
C_{10b}**	*NaCl-***α5.5**-4.3	28.10 ± 5.03 (4)	n.d.	-	***3.8***

† **α symbol** followed by a **number** indicates α-CD concentration; underlined number indicates pH value (detailed information in Table 1).
[a] experimental data, from equation 1
[b] calculated from equation 4
[c] calculated *vs* C_0, using equation 5
[n.c.] not calculable
[n.d.] not determined
* statistically different from C_0, $p<0.05$ (Student's t test)
** statistically different from C_0, $p<0.01$ (Student's t test)

3.1.1 Role of pH, EDTA and benzalkonium chloride

First of all, the role of pH (4.2 *vs* 7.4) was evaluated: cysteamine was dissolved in 0.9% NaCl and the pH was bought to 4. (C_{3a}), or 7.4 (C_{3b}). As shown in Figure 2, the permeation is very slow and low at pH 4.2 (C_{3a}), where cysteamine logD is -3.12 and 99.98% of the molecule is positively charged [19], while a measurable flux is present at pH 7.4 (C_{3b}) where cysteamine logD increases to -2.36, due to the presence of 20% amphiphilic form and 0.33% of unionized form [19]. The apparent permeability coefficient value obtained at pH 7.4 ($2.60\pm0.75*10^{-6}$ cm/s) was compared with the value calculated using a computational model recently set up and validated [28], built using the total hydrogen bonding capacity (HB_{tot}) and the distribution coefficient at pH 7.4 ($logD_{7.4}$) as descriptors:

$$logP_{app}=-3.885-0.183(HB_{tot})+0.277(logD_{7.4})$$ **equation 6** [28].

The calculated value ($5.36*10^{-6}$ cm/s) is similar to the experimental one, demonstrating the predictability of the model (although built using rabbit data) and confirming that molecular weight (not present in the equation) has a limited role in the definition of trans-corneal permeability, at least for low MW compounds [28]. The main barrier to cysteamine diffusion across the cornea is the lipophilic epithelium [20], as can be observed by comparing the

permeability value across the full thickness cornea and de-epithelialized cornea reported in Table 2.

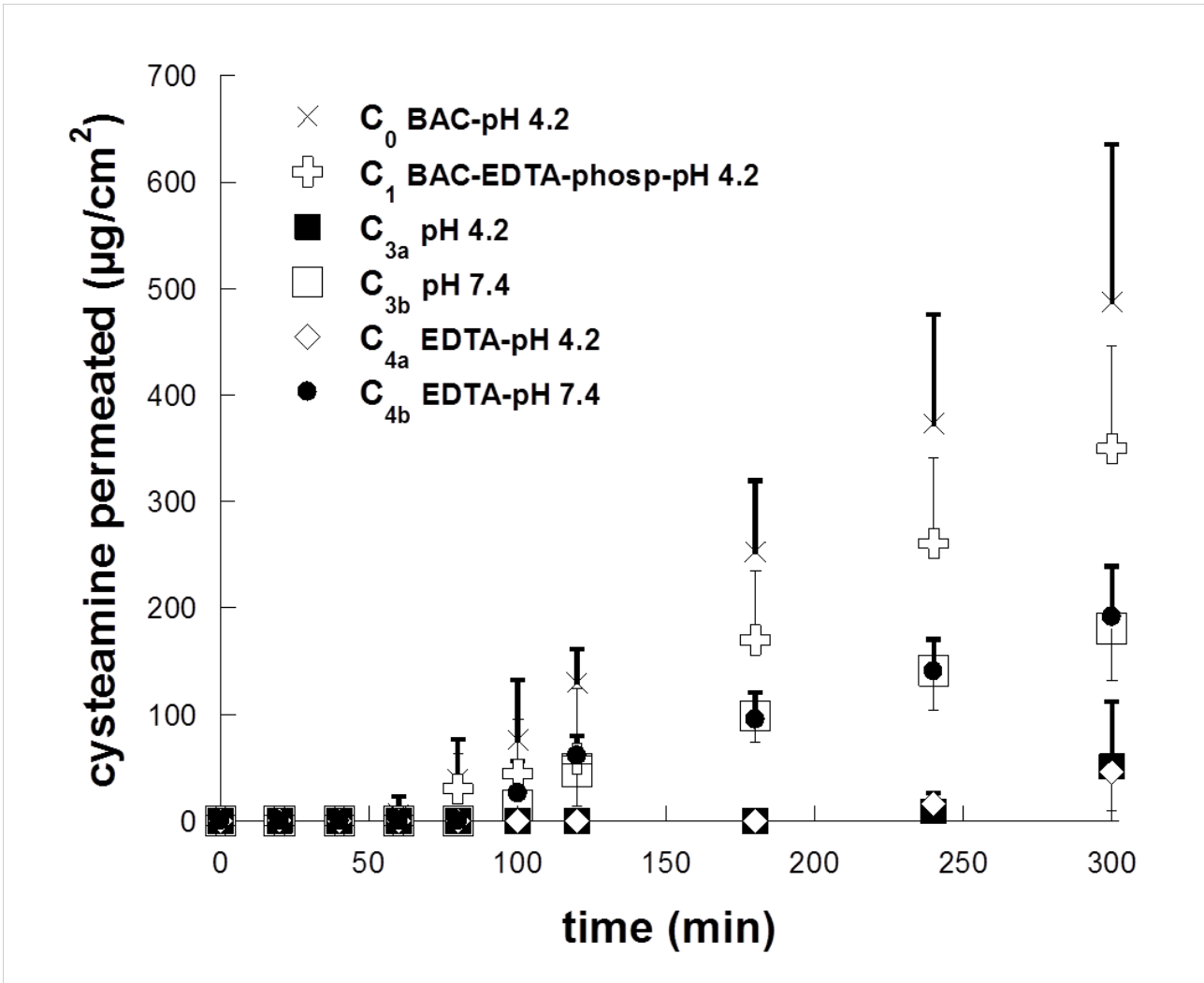


Figure 2. Permeation profiles of cysteamine across porcine cornea. 0.44% cysteamine was dissolved in different vehicles: C_0 (pH 4.2, BAC), C_1 (buffered pH 4.2, BAC, EDTA), C_{3a} (pH 4.2), C_{3b} (pH 7.4), C_{4a} (pH 4.2, EDTA) and C_{4b} (pH 7.4, EDTA) (mean values±sd; 3<n<7)

EDTA was then added to pH 4.2 (C_{4a}) and 7.4 (C_{4b}) solutions to evaluate its potential enhancer effect. The literature data on EDTA are controversial: some authors [29] [30] [31] reported an enhancer effect due to chelation of calcium ions involved in the opening of tight junctions [32]. On the contrary, other authors found no effect of EDTA in the trans-corneal permeation of either hydrophilic [33] or lipophilic [34] [35] [30] drugs. The permeation profiles obtained with cysteamine are superimposed with the respective controls (Figure 2), showing the absence of enhancing effect at both pH 4.2 ($EDTA^{2-}$; C_{3a} *vs* C_{4a}) and 7.4 ($EDTA^{4-}$; C_{3b} *vs* C_{4b}).

The commercial product Cystaran™ (here reproduced by C_0) contains the antimicrobial agent benzalkonium chloride, also well known as permeation enhancer for trans-corneal administration [36]. The enhancement effect of BAC can be seen from the permeation profiles in Figure 2: the amount permeated after 5 hours increased approximately 10 times compared to the control (C_{3a}, no BAC) and the lag time (here defined as the first timepoint at which the drug is detected in the receptor compartment) significantly decreased from 4 to 1 h. It is worth mentioning that the presence of a permeation enhancer in the commercial product is required because in patients affected by ocular cystinosis the barrier function of the corneal epithelium is preserved. In fact, even if corneal thickness appears higher, due to disregulation of corneal water balance and consequent swelling of the stroma, the epithelium remains intact [37].

Due to the low solution stability of cysteamine at neutral pH, the commercial eye-drop Cystaran™ has a pH of 4.2 (C_0, (Table 1)): however, because it is an unbuffered solution, its pH value increased during the experiment from the initial value of 4.2 to 6 (after 2 hours) and to 6.4 (after 5 hours) (C_0, Table 3).

Table 3. pH of donor solutions during permeation experiments (mean values ± sd; 3<n7)

		pH		
vehicle	composition†	time 0	time 2h	time 5h
C0	*NaCl-BAC-4.2*	4.30 ± 0.08	6.02 ± 0.06	6.37 ± 0.11
C1	*BAC-EDTA-phosp-4.2*	4.23 ± 0.02	4.46 ± 0.04	4.64 ± 0.13
C3a	*NaCl-4.2*	4.35 ± 0.02	5.52 ± 0.05	5.95 ± 0.33
C3b	*NaCl-7.4*	7.39 ± 0.04	7.26 ± 0.05	7.25 ± 0.05
C4a	*NaCl-EDTA-4.2*	4.19 ± 0.03	4.80 ± 0.05	5.01 ± 0.10
C4b	*NaCl-EDTA-7.4*	7.42 ± 0.02	7.25 ± 0.06	7.21 ± 0.07
C9c	*NaCl-EDTA-**α4.9**-4.2*	4.34 ± 0.00	5.60 ± 0.10	6.28 ± 0.06
C9d	*NaCl-EDTA-phosp-**α4.9**-4.2*	4.15 ± 0.00	4.54 ± 0.07	4.99 ± 0.05

† underlined number indicates pH value; **α symbol** followed by a **number** indicates α-CD concentration; (for more detailed information see Table 1).

This pH shift probably happens also *in vivo,* where the product is diluted with the lacrimal fluid (pH 7.4) and is in contact with the ocular tissues, leading to a change (decrease) of drug ionization that can increase significantly its corneal permeability, as previously observed.

Despite its efficacy, Cystaran™ has still a problem of stability: the product has to be stored at -20°C and, after opening, it should be used within 1 week, keeping it between +2°C and

+25°C [8]. With the aim of improving drug stability, Tsilou and colleagues modified the formulation using EDTA and sodium phosphate (C_1, Table 1): however, the clinical evaluation demonstrated that this formulation was not active anymore [15]. A possible explanation of the reduction of efficacy can be the presence of phosphate in C_1, leading to a lower variation of pH in the solution (from 4.2 to 4.5 after 5 hours, see Table 3), compared to the unbuffered solution C_0. The permeation data here collected with this formulation (C_1) cannot totally explain the clinical results, because the permeation profile was lower but not statistically different from C_0 (see Figure 2). However, the pH shifts observed *in vitro* are probably under-estimated compared to what happens *in vivo*, where a dilution with lacrimal fluid takes place and, above all, the contact area between formulation and ocular tissues is much higher.

3.1.2 Role of alpha-cyclodextrin

α-CD was chosen to improve the trans-corneal permeation of cysteamine. α-CD has been reported to improve ocular delivery of lipophilic drugs, such as cyclosporin [38] and Δ9-tetrahydrocannobinol [39] increasing their solubility thus overcoming the aqueous layer that normally prevents the interaction between drug and cornea [40]. CDs may also act as proper permeation enhancer by extracting lipidic components of cellular membrane, as demonstrated for α-CD on isolated erythrocytes [41] and epithelial cells of bovine cornea [42]. This mechanism is negligible for lipophilic compounds, being inherently able to diffuse across membranes, but could be relevant for hydrophilic drugs such as cysteamine.

Therefore, vehicles containing α-CD in concentrations included between 3 and 5.5% were evaluated. The profiles obtained, reported in Figure 3a, show an important enhancing effect, proportional to concentration (Figure 4, full squares): using 5.5% α-CD of it is possible to increase the amount permeated up to 20 times compared to the control (C_{3a}). Also with this enhancer, a reduction of the lag time can be appreciated, but still 60 minutes are necessary to detect the drug in the receiver solution.

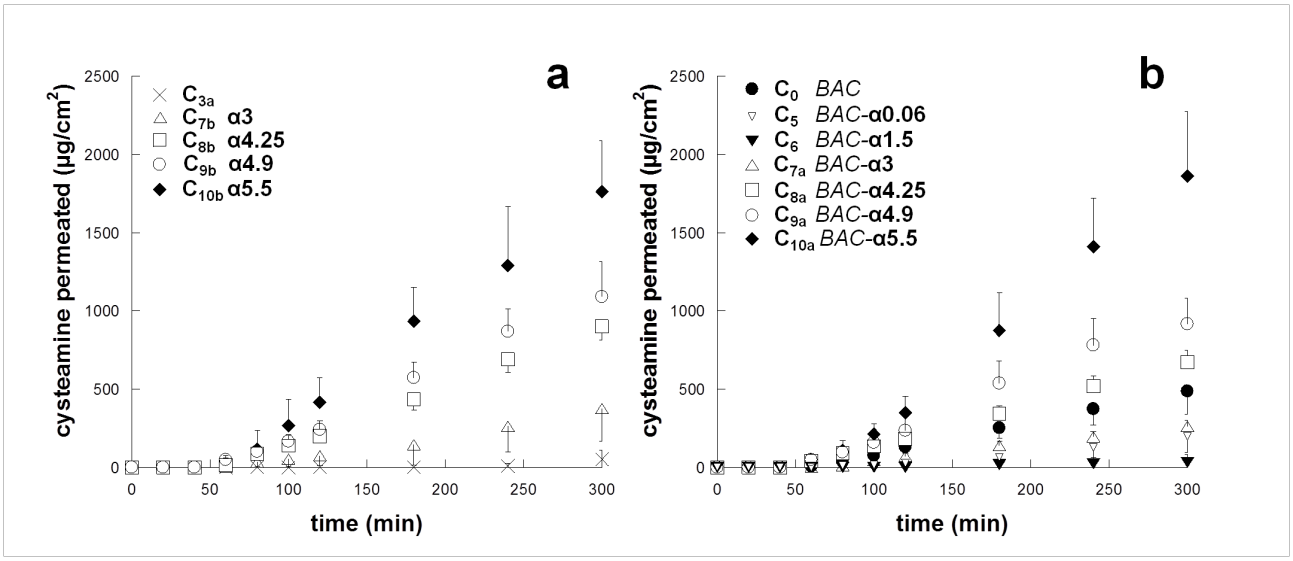


Figure 3. Cysteamine permeation profiles through full-thickness cornea starting from vehicles containing α-CD (a) and α-CD and BAC (b), compared to reference vehicles (C_{3a} and C_0, respectively). (mean values±sd; 3<n<7)

The results demonstrated that relatively high concentrations are necessary to promote cysteamine diffusion across the cornea; 3% of CD can match the performance of 0.01% BAC. Siefert and Keipert [42] obtained similar results: the uptake of pilocarpine hydrochloride in bovine cornea was not influenced by 0.5% α-CD, while higher concentrations (8.54 and 13.92%) had a dramatic effect. The hypothesized mechanism was extraction of lipidic fractions from epithelial cells [42]. Nonetheless, the efficacy of α-CD is also molecule-dependent, since Morrison and colleagues found that α-CDs were not able to promote riboflavin diffusion across bovine cornea *ex vivo,* as β and HPβ-CD did [43].

Cyclodextrins were then associated with BAC to evaluate the possibility to obtain a synergistic effect, at low cyclodextrin concentration. The data, reported in Figure 3b and Figure 4 (void squares) show that 0.06% CD (BAC:α-CD molar ratio, 1:2) reduced the enhancing properties of BAC and 1.5% CD completely suppressed it. Indeed, the interaction between CDs and BAC has been described [44, 45] and we can hypothesize that cyclodextrin prevents BAC effect on corneal epithelium. Only when CD concentration is 3% or higher, the permeation is restored, due to the enhancing effect of CDs. The results obtained with the vehicles containing the association of BAC and CD are not statistically different from the ones obtained with CD alones, with the exception of 4.25% α-CD. In fact, unexpectedly, C_{8b} is statistically higher than C_{8a} ($p<0.01$), even if the reason is unknown.

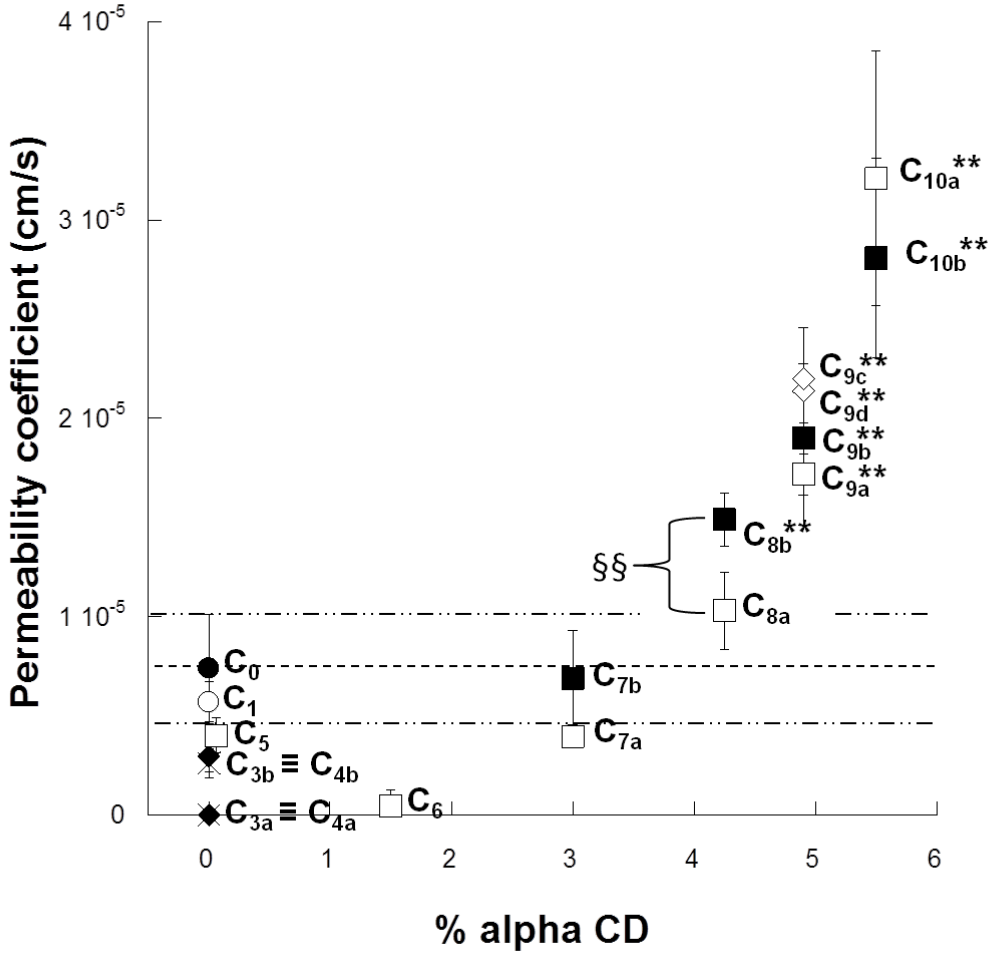


Figure 4. Permeability coefficients through full-thickness porcine cornea as a function of α-CD concentration. Full squares (■) represent vehicles containing only NaCl and CDs, void squares (□) represent vehicles containing NaCl, CDs and BAC, open diamond (◇) represent vehicles containing NaCl, CDs and EDTA. (mean values±sd; 3<n<7). The detailed composition is illustrated in Table 1. Asterisks refer to statistical difference from the commercial formulation C_0 **, p<0.01; Student's t test); §§ refers to statistical difference between C_{8a} and C_{8b} (p<0.01; Student's t test).

3.2 Stability studies

The other challenge related to cysteamine administration is the poor stability of cysteamine hydrochloride in aqueous solution. For this reason, we have at first evaluated the stability pattern of three known formulations, examples of low (C_2; pH 7.4), average (C_0; pH 4.2;) and good stability (C_1, pH 4.2; stable but ineffective). The results (Figure 5) confirmed: 1) the quantitative conversion of cysteamine into cystamine; 2) the pH dependence of this phenomenon: at pH 7.4 the oxidation is extremely rapid due to the presence of ionized thiol

groups; 3) the temperature dependence of oxidation; 4) the very good stability of C_1 (as previously demonstrated [15]) due to the presence of acidic pH, EDTA and sodium phosphate.

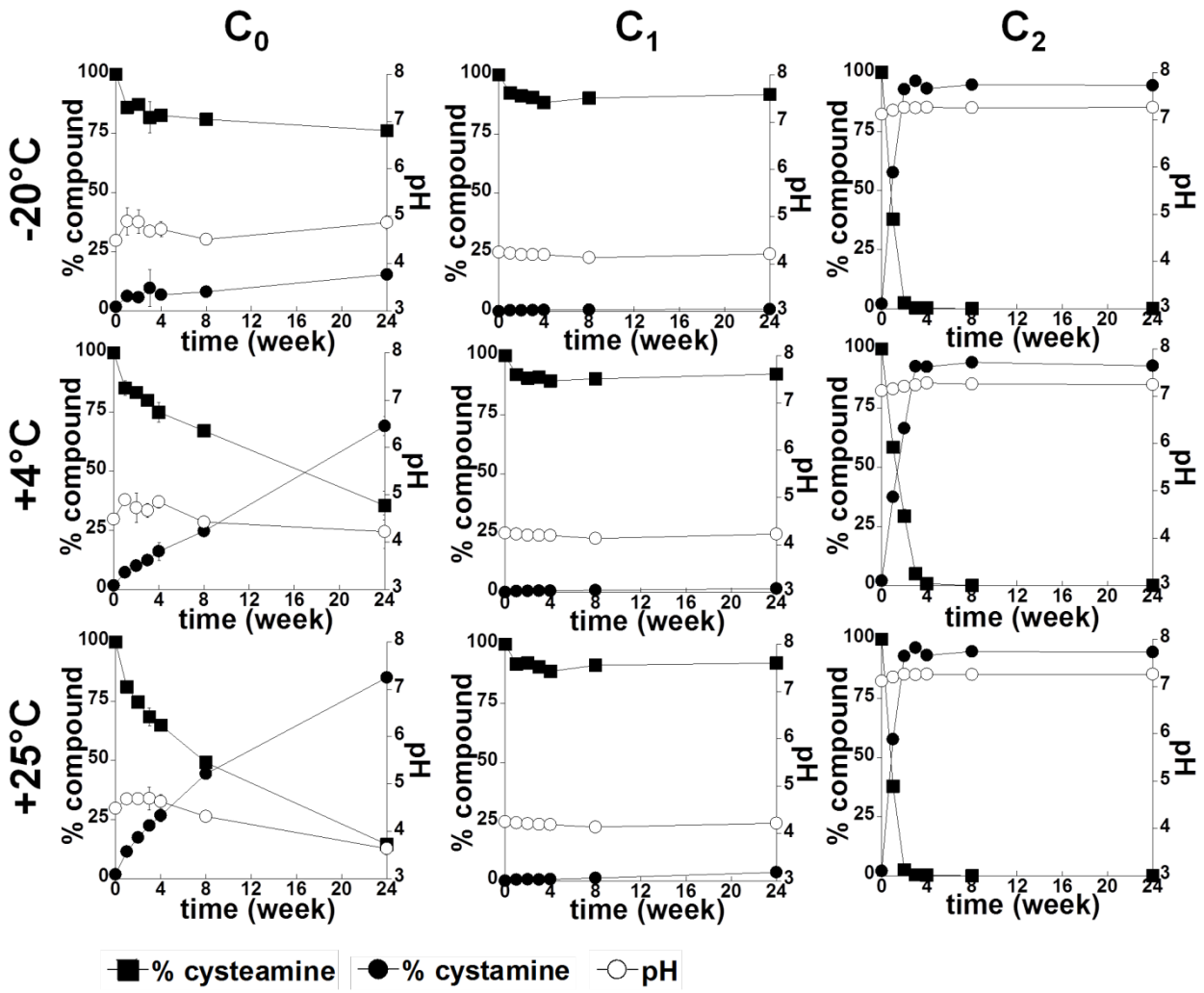


Figure 5. Percentage of cysteamine (full square) and cystamine (full circle) recovered in C_0 (*NaCl-BAC-4.2*), C_1 (*BAC-EDTA-phosp-4.2*) and C_2 (*NaCl-EDTA-hepes-7.4*) stored at -20°C, +4°C and +25°C over 24 weeks stability studies; void circles represent pH value. (mean values±sd; n=3).

Then, CD-containing vehicles were analyzed. In principle, cysteamine can interact with cyclodextrin surface; this interaction is reported only in the solid state [46] but is also possible in aqueous solution, as found for other hydrophilic compounds [47]. However, as can be seen in Table 4, neither the presence of CD alone (C_{9b}), nor its association with BAC (C_{7a}) increased cysteamine stability.

Table 4. Percentage of cysteamine recovered from solutions at pH 4.2 C_{7a}, C_{9b}, C_{9c}, C_{9d} stored at -20°C, +4°C and +25°C over 4 or 24 weeks stability studies. (mean values ± sd; n=3)

vehicle†	*temperature*	*Week 4*	*Week 8*	*Week 24*
C_{7a} *NaCl-BAC* **α3** 4.2	-20°C	83.1 ± 17.0	n.d.	n.d.
	+4°C	44.6 ± 8.5	n.d.	n.d.
	+25°C	32.1 ± 6.8	n.d.	n.d.
	temperature	***Week 5***	***Week 8***	***Week 24***
C_{9b} *NaCl* **α4.9** 4.2	-20°C	54.7 ± 4.8	n.d.	n.d.
	+4°C	62.3 ± 12.2	n.d.	n.d.
	+25°C	39.1 ± 7.5	n.d.	n.d.
	temperature	***Week 4***	***Week 8***	***Week 24***
C_{9c} *NaCl-EDTA* **α4.9** 4.2	-20°C	98.6 ± 0.4	n.d.	n.d.
	+4°C	95.7 ± 2.0	n.d.	n.d.
	+25°C	89.6 ± 2.4	n.d.	n.d.
	temperature	***Week 4***	***Week 8***	***Week 24***
C_{9d} *NaCl-EDTA-phosp* **α4.9** 4.2	-20°C	97.0 ± 0.8	98.5 ± 2.0	98.8 ± 0.7
	+4°C	97.0 ± 0.7	98.4 ± 0.8	95.9 ± 3.0
	+25°C	95.7 ± 0.6	95.5 ± 1.7	90.7 ± 0.7

† underlined number indicates pH value; **α symbol** followed by a **number** indicates α-CD concentration; (for more detailed information see Table 1).
n.d. not determined

To try to stabilize CD formulations, EDTA alone or in combination with sodium phosphate was added to C_{9b}. The obtained solutions (C_{9c} and C_{9d}) showed a behavior comparable to C_1 (Figure 5), demonstrating the crucial role of EDTA in preventing cysteamine oxidation and that α-cyclodextrin does not affect the stability in a negative way. Apparently, the presence of phosphate is not necessary for the stability, but these data are available only up to one month and should be confirmed with a longer-term validation.

These formulations (C_{9c} and C_{9d}) were also evaluated for their performance in terms of cysteamine permeation across the cornea: the results suggest that neither EDTA, nor the association between EDTA and phosphate suppressed the enhancing effect of α-CD, because the data were comparable to those achieved from C_{9b} (Figure 5). However, also in this case it is important to underline that, in the presence of phosphate, no pH shift was recorded in the donor solution during the permeation experiment (Table 3), and for the above mentioned reasons the formulation C_{9d} should be preferred.

3.3 DLS analysis

Some considerations have to be done concerning the high concentration of α-CD, causing opalescence in the formulations and, in case of concentration higher than 3%, the formation of a light and readily re-dispersible sediment. It is known that two or more CD units are able to form water soluble aggregates that increase in size by increasing CD concentration [48] [49]; and these aggregates can also interact with drugs forming non-inclusion complexes or micelles [50]. α-CD forms in water aggregates ranging in size between 6 and 155 nm, the dimension tends to increase up to 1 μm when a drug is added to the solution [51].

The DLS analysis of the solution containing 4.9% α-CD in water showed aggregates with a diameter between 150 and 200 nm while C_{9d} vehicle with and without 0.44% cysteamine contained larger aggregates ranging in size from 400 to 800 nm. In principle, the presence of aggregates should not be a concern, being the size much smaller than 25 μm and thus non irritant for the ocular surface [52]. Additionally, the sediment disperses readily when the container is shaken, and the size of the dispersed particles did not change after 6 months of storage. It is also worth mentioning that particles of nanometric size could, *in vivo*, increase the residence time on the ocular surface and, as a consequence, drug bioavailability [51, 53].

3.4 HET-CAM assay

The HET-CAM assay was set up to evaluate the possible irritating effect on the ocular conjunctiva. The HET-CAM assay is based on an organotypic model and is in close agreement, especially for mild and non-irritating substances, with *in vivo* results obtained on rabbit (Draize test), even if the latter is never completely replaceable [54]. The test is commonly used for the screening of the irritant potential of chemicals, eye-drops [47], but also gels [55] or pH and thermo-responsive *in situ* gels [56].

The test was performed on solutions containing 5.5% α-CD, with and without 0.44% cysteamine and/or 0.01% BAC at pH of 4.2. The results underline the absence of irritation (IS=0, data not shown). This results is also supported by literature data of *in vivo* rabbit tests showed that a solution containing 8% α-CD was practically non-irritating (even in presence of cyclosporine as active compound) [38], as also demonstrated by other authors [47, 57].

4 CONCLUSION

In the present paper, for the first time, the permeation of cysteamine across the cornea was studied. The formulation factors influencing the permeation were the pH value and the presence of permeation enhancers, but also the vehicle buffering capacity – or better the absence of it - seems to play a very important role.

The data here collected demonstrated that the presence of benzalkonium chloride in the commercial eye-drops is necessary to obtain cornea penetration. We have evaluated α-CD as an alternative penetration enhancer and demonstrated its ability to promote the trans-corneal diffusion of cysteamine. In particular, we have obtained, at a 5.5% α-CD concentration, a 4-fold higher penetration compared to the BAC-containing commercial eye-drops. This result could in principle translate into an higher efficacy and/or into the possibility of reducing the administration frequency, increasing patient's compliance. Furthermore, by associating EDTA to the α-CD vehicle, it is possible to obtain a good cysteamine stability profile, facing one of the other crucial cysteamine issue. Instead of the antimicrobial agent, particularly critical in case of a chronic ocular diseases with high administration need, preservative-free solutions and single-dose devices could be used.

The irritation potential of this formulation has been evaluated using the HET-CAM assay, however the data have to be confirmed with an *in vivo* test and completed with further evaluation of chronic tolerability. Finally, to prove the ability of the formulation to reduce and prevent the formation of cystine crystals, *in vivo* studies involving *CTNS* knockout mouse model should be performed. As previously mentioned, also other cyclodextrins, such as beta, hydroxypropyl beta and gamma, are reported in the literature as trans-corneal penetration enhancers and could represent interesting alternatives for the improvement of cysteamine bioavailability.

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REFERENCES

[1] FDA, Orphan designation of cysteamine hydrochloride for the treatment of cystinosis Treatment of corneal cystine crystal accumulation in cystinosis patients. , in, http://www.accessdata.fda.gov/scripts/opdlisting/oopd/OOPD_Results_2.cfm?Index_Number=105997 (accessed March, 2016), 1997.
[2] EMA, EMEA/COMP/552795/2014 - Public summary of opinion on orphan designation Cysteamine hydrochloride for the treatment of cystinosis (EU/3/14/1341), in, http://www.ema.europa.eu/docs/en_GB/document_library/Orphan_designation/2014/11/WC500177177.pdf (accessed March, 2016), 2014.
[3] G. Nesterova, W.A. Gahl, Cystinosis, in: R.A. Pagon, M.P. Adam, H.H. Ardinger, S.E. Wallace, A. Amemiya, L.J.H. Bean, T.D. Bird, C.R. Dolan, C.T. Fong, R.J.H. Smith, K. Stephens (Eds.) GeneReviews [Internet], University of Washington, Seattle, Seattle WA, 2014.
[4] F. Shams, I. Livingstone, D. Oladiwura, K. Ramaesh, Treatment of corneal cystine crystal accumulation in patients with cystinosis, Clinical ophthalmology (Auckland, N.Z.), 8 (2014) 2077-2084.
[5] A. Labbé, P. Niaudet, C. Loirat, M. Charbit, G. Guest, C. Baudouin, In Vivo Confocal Microscopy and Anterior Segment Optical Coherence Tomography Analysis of the Cornea in Nephropathic Cystinosis, Ophthalmology, 116 (2009) 870-876.
[6] FDA, Cystaran NDA approval ID 3198343, in, http://www.accessdata.fda.gov/drugsatfda_docs/appletter/2012/200740Orig1s000ltr.pdf (accessed March, 2016), 2012.
[7] F. Iwata, E.M. Kuehl, G.F. Reed, L.M. McCain, W.A. Gahl, M.I. Kaiser-Kupfer, A Randomized Clinical Trial of Topical Cysteamine Disulfide (Cystamine) versus Free Thiol (Cysteamine) in the Treatment of Corneal Cystine Crystals in Cystinosis, Molecular Genetics and Metabolism, 64 (1998) 237-242.
[8] Sigma-Tau Pharmaceuticals Inc., Cystaran™ (cysteamine ophthalmic solution) 0.44% - Technical Sheet/package insert, in, Gaithersburg, MD 20878 2012.
[9] S. Bozdag, K. Gumus, O. Gumus, N. Unlu, Formulation and in vitro evaluation of cysteamine hydrochloride viscous solutions for the treatment of corneal cystinosis, Eur J Pharm Biopharm, 70 (2008) 260-269.
[10] B. Buchan, G. Kay, A. Heneghan, K.H. Matthews, D. Cairns, Gel formulations for treatment of the ophthalmic complications in cystinosis, Int J Pharm, 392 (2010) 192-197.
[11] A. Labbé, C. Baudouin, G. Deschênes, C. Loirat, M. Charbit, G. Guest, P. Niaudet, A new gel formulation of topical cysteamine for the treatment of corneal cystine crystals in cystinosis: The Cystadrops OCT-1 study, Molecular Genetics and Metabolism, 111 (2014) 314-320.
[12] U. Sundralingam, O. Rabiu, W. Van't Hoff, K. Nischal, C. Tuleu, Formulating cysteamine polymeric systems for the treatment of corneal crystals in cystinosis, in: 3rd Annual Conference - European Pediatric Formulation Initiative, Berlin, 2010.
[13] K.H. Hsu, R.C. Fentzke, A. Chauhan, Feasibility of corneal drug delivery of cysteamine using vitamin E modified silicone hydrogel contact lenses, Eur J Pharm Biopharm, 85 (2013) 531-540.
[14] J. Simpson, Sustaine-Release Cysteamine Nanowafer Drug Delivery fo Corneal Cystinosis, in: C.F. UK (Ed.) 8th International Cystinosis Congress Beyond Borders, Manchester, UK, 2014.
[15] E.T. Tsilou, D. Thompson, A.S. Lindblad, G.F. Reed, B. Rubin, W. Gahl, J. Thoene, M. Del Monte, J.A. Schneider, D.B. Granet, M.I. Kaiser-Kupfer, A multicentre randomised double masked clinical trial of a new formulation of topical cysteamine for the treatment of corneal cystine crystals in cystinosis, Br J Ophthalmol, 87 (2003) 28-31.
[16] M. Santaniello, L. Critelli, G. Quattrociocchi, A. Longo, W. Cabri, Reconstituted ocular gel for cystinosis treatment Journal of Drug Delivery Science and Technology 21 (2011) 273-277.
[17] EMA, EMA/495737/2013 - Questions and answers on benzalkonium chloride in the context of the revision of the guideline on "Excipients in the label and package leaflet of medicinal products for human use" (CPMP/463/00), in, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2014/02/WC500162031.pdf (accessed June, 2016), 2014.
[18] P.D. Freeman, M.Y. Kahook, Preservatives in topical ophthalmic medications: historical and clinical perspectives, Expert Review of Ophthalmology, 4 (2009) 59-64.

[19] Advanced Chemistry Development Inc., ACD/Percepta, Version 2015, Pack 2, in: Toronto, On, Canada, www.acd.labs.com, 2015.
[20] S. Pescina, P. Govoni, A. Potenza, C. Padula, P. Santi, S. Nicoli, Development of a convenient ex vivo model for the study of the transcorneal permeation of drugs: histological and permeability evaluation, J Pharm Sci, 104 (2015) 63-71.
[21] M.R. Prausnitz, J.S. Noonan, Permeability of cornea, sclera, and conjunctiva: a literature analysis for drug delivery to the eye, J Pharm Sci, 87 (1998) 1479-1488.
[22] ICCVAM, Recommended test method protocol: Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) Test Method; NIH Publication No. 10-7553, in, 2010.
[23] EMA, Cysteamine Hydrochloride for nephropathic Cystinosis, open-label Phase III pivotal study (EudraCT number 2009-012564-13), in, European Clinical Trials Register, 2013.
[24] K.R. Kenyon, J.A. Sensenbrenner, Electron microscopy of cornea and conjunctiva in childhood cystinosis, American journal of ophthalmology, 78 (1974) 68-76.
[25] J.E. Dominy, Jr., C.R. Simmons, L.L. Hirschberger, J. Hwang, R.M. Coloso, M.H. Stipanuk, Discovery and characterization of a second mammalian thiol dioxygenase, cysteamine dioxygenase, The Journal of biological chemistry, 282 (2007) 25189-25198.
[26] R. Coloso, L. Hirschberger, J. Dominy, J.-I. Lee, M. Stipanuk, Cysteamine Dioxygenase: Evidence for the Physiological Conversion of Cysteamine to Hypotaurine in Rat and Mouse Tissues, in: S. Oja, P. Saransaari (Eds.) Taurine 6, Springer US, 2006, pp. 25-36.
[27] M.J. Wilmer, L.A.J. Kluijtmans, T.J. van der Velden, P.H. Willems, P.G. Scheffer, R. Masereeuw, L.A. Monnens, L.P. van den Heuvel, E.N. Levtchenko, Cysteamine restores glutathione redox status in cultured cystinotic proximal tubular epithelial cells, Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1812 (2011) 643-651.
[28] H. Kidron, K.S. Vellonen, E.M. del Amo, A. Tissari, A. Urtti, Prediction of the corneal permeability of drug-like compounds, Pharm Res, 27 (2010) 1398-1407.
[29] M. Ahuja, A.S. Dhake, D.K. Majumdar, Effect of formulation factors on in-vitro permeation of diclofenac from experimental and marketed aqueous eye drops through excised goat cornea, Yakugaku Zasshi, 126 (2006) 1369-1375.
[30] G.M. Grass, R.W. Wood, J.R. Robinson, Effects of calcium chelating agents on corneal permeability, Invest Ophthalmol Vis Sci, 26 (1985) 110-113.
[31] P.W.J. Morrison, V.V. Khutoryanskiy, Enhancement in corneal permeability of riboflavin using calcium sequestering compounds, Int J Pharm, 472 (2014) 56-64.
[32] Y. Rojanasakul, J. Liaw, J.R. Robinson, Mechanisms of action of some penetration enhancers in the cornea: Laser scanning confocal microscopic and electrophysiology studies, Int J Pharm, 66 (1990) 131-142.
[33] M.F. Saettone, P. Chetoni, R. Cerbai, G. Mazzanti, L. Braghiroli, Evaluation of ocular permeation enhancers: in vitro effects on corneal transport of four β-blockers, and in vitro/in vivo toxic activity, Int J Pharm, 142 (1996) 103-113.
[34] P. Ashton, W. Wang, V.H. Lee, Location of penetration and metabolic barriers to levobunolol in the corneal epithelium of the pigmented rabbit, The Journal of pharmacology and experimental therapeutics, 259 (1991) 719-724.
[35] C. Madhu, P.J. Rix, M.J. Shackleton, T.G. Nguyen, D.D. Tang-Liu, Effect of benzalkonium chloride/EDTA on the ocular bioavailability of ketorolac tromethamine following ocular instillation to normal and de-epithelialized corneas of rabbits, J Pharm Sci, 85 (1996) 415-418.
[36] N.L. Burstein, Preservative alteration of corneal permeability in humans and rabbits, Invest Ophthalmol Vis Sci, 25 (1984) 1453-1457.
[37] B. Katz, R.B. Melles, J.A. Schneider, N.A. Rao, Corneal thickness in nephropathic cystinosis, Br J Ophthalmol, 73 (1989) 665-668.
[38] A. Kanai, R.M. Alba, T. Takano, C. Kobayashi, A. Nakajima, K. Kurihara, T. Yokoyama, M. Fukami, The effect on the cornea of alpha cyclodextrin vehicle for cyclosporin eye drops, Transplantation proceedings, 21 (1989) 3150-3152.
[39] E.C. Kearse, K. Green, Effect of vehicle upon in vitro transcorneal permeability and intracorneal content of Delta9-tetrahydrocannabinol, Current eye research, 20 (2000) 496-501.

[40] M. Masson, T. Loftsson, G. Masson, E. Stefansson, Cyclodextrins as permeation enhancers: some theoretical evaluations and in vitro testing, Journal of controlled release : official journal of the Controlled Release Society, 59 (1999) 107-118.
[41] Y. Ohtani, T. Irie, K. Uekama, K. Fukunaga, J. Pitha, Differential effects of α-, β- and γ-cyclodextrins on human erythrocytes, European Journal of Biochemistry, 186 (1989) 17-22.
[42] B. Siefert, S. Keipert, Influence of alpha-cyclodextrin and hydroxyalkylated beta-cyclodextrin derivatives on the in vitro corneal uptake and permeation of aqueous pilocarpine-HCl solutions, J Pharm Sci, 86 (1997) 716-720.
[43] P.W.J. Morrison, C.J. Connon, V.V. Khutoryanskiy, Cyclodextrin-mediated enhancement of riboflavin solubility and corneal permeability, Molecular Pharmaceutics, 10 (2013) 756-762.
[44] B. Malaekeh-Nikouei, B.S. Fazly Bazzaz, V. Soheili, K. Mohammadian, Problems in Ophthalmic Drug Delivery: Evaluation of the Interaction Between Preservatives and Cyclodextrins, Jundishapur J Microbiol, 6 (2013) e6333.
[45] T. Loftsson, Ó. Stefánsdóttir, H. Friðriksdóttir, Ö. Guðmundsson, Interactions between preservatives and 2-hydroxypropyl-β-cyclodextrin, Drug Development and Industrial Pharmacy, 18 (1992) 1477-1484.
[46] M. Lahiani-Skiba, Y. Boulet, I. Youm, F. Bounoure, P. Vérité, P. Arnaud, M. Skiba, Interaction between hydrophilic drug and α-cyclodextrins: physico-chemical aspects, J Incl Phenom Macrocycl Chem, 57 (2007) 211-217.
[47] S. Keipert, J. Fedder, A. Bohm, B. Hanke, Interactions between cyclodextrins and pilocarpine — As an example of a hydrophilic drug, Int J Pharm, 142 (1996) 153-162.
[48] P. Jansook, S.V. Kurkov, T. Loftsson, Cyclodextrins as solubilizers: formation of complex aggregates, J Pharm Sci, 99 (2010) 719-729.
[49] A. Wu, X. Shen, Y. He, Investigation on γ-cyclodextrin nanotube induced by -diphenylbenzidine molecule, Journal of Colloid and Interface Science, 297 (2006) 525-533.
[50] T. Loftsson, M. Masson, M.E. Brewster, Self-association of cyclodextrins and cyclodextrin complexes, J Pharm Sci, 93 (2004) 1091-1099.
[51] S. Johannsdottir, P. Jansook, E. Stefansson, T. Loftsson, Development of a cyclodextrin-based aqueous cyclosporin A eye drop formulations, Int J Pharm, 493 (2015) 86-95.
[52] Council of Europe, European Pharmacopoeia, 8th ed., vol I, Dosage Forms, EDQM, 67075 Strasbourg Cedex, France, 2013.
[53] S. Pescina, F. Sonvico, P. Santi, S. Nicoli, Therapeutics and carriers: the dual role of proteins in nanoparticles for ocular delivery, Current topics in medicinal chemistry, 15 (2015) 369-385.
[54] M. Balls, P.A. Botham, L.H. Bruner, H. Spielmann, The EC/HO international validation study on alternatives to the draize eye irritation test, Toxicol In Vitro, 9 (1995) 871-929.
[55] B. McKenzie, G. Kay, K.H. Matthews, R.M. Knott, D. Cairns, The hen's egg chorioallantoic membrane (HET-CAM) test to predict the ophthalmic irritation potential of a cysteamine-containing gel: Quantification using Photoshop and ImageJ, Int J Pharm, 490 (2015) 1-8.
[56] H. Gupta, S. Jain, R. Mathur, P. Mishra, A.K. Mishra, T. Velpandian, Sustained Ocular Drug Delivery from a Temperature and pH Triggered Novel In Situ Gel System, Drug Delivery, 14 (2007) 507-515.
[57] Y. Sasamoto, S. Hirose, S. Ohno, K. Onoe, H. Matsuda, Topical application of ciclosporin ophthalmic solution containing alpha-cyclodextrin in experimental uveitis, Ophthalmologica. Journal international d'ophtalmologie. International journal of ophthalmology. Zeitschrift fur Augenheilkunde, 203 (1991) 118-125.