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Validation of a device for transcorneal drug permeation measure

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ABSTRACT

A model of a corneal apparatus for the study of drug permeation across the corneal tissues is evaluated. The instrument was designed to measure drug transport in living rabbit corneas, which are placed hermetically into a chamber, separating it into two compartments (epithelial and endothelial). The device also allows the measurement and recording of the corneal potential, providing information on the vitality of the cornea.

The validation of the instrument was carried out by introducing variable amounts of fluorescein into the epithelial compartment of the chamber, and measuring the concentration of the dye by the fluorescence obtained in the endothelial compartment. The fluorescein concentration in the endothelial compartment increased exponentially over time, with a good correlation between the epithelial and endothelial concentrations. The repeatability of the measurements is strongly influenced by the maintenance and regularity of the initial concentration, the time that has passed from the beginning of the experiment, and the corneal potential (vitality of the cornea). Less important are slight modifications to temperature, the agitation system and hydrostatic pressure (robustness).

The proposed experimental system shows good precision when measuring the permeation through rabbit cornea at different drug concentrations, and good repeatability and intermediate precision with non-significant differences among operators in repeated experiments. It is a good approach for comparing corneal transport of different formulations of the same drug.

To test the method with real samples, different formulations of the NSAID sodium diclofenac where assayed. Corneal permeation was remarkably enhanced when diclofenac was formulated as a β-cyclodextrin complex. Other additives, such as the surfactants used in commercial eye drops, also enhanced corneal drug transport.

1. Introduction

In recent years numerous studies have appeared related to transport phenomena in different kinds of tissue. The cornea provides a natural barrier to drug transport of topical drugs. In warm-blooded animals the cornea seems to be particularly well suited for such "ex vivo" studies. There is no blood circulation, and properties such as optical transparency, water-content, and thickness, besides the measurable electrical phenomena, offer good opportunities for following the state of the tissue.

The bioavailability of drugs administered by the topical route for the treatment of ocular diseases depends on the nature of the active principle and the pharmaceutical form, as well as the special anatomophysiological characteristics of the ocular apparatus. There are three areas (precorneal, corneal and postcorneal) that the drug must cross to exert its action in the anterior or posterior chamber of the eye.

To be effective, most drugs must penetrate across the eye's tissue barriers (i.e. cornea) to reach therapeutic targets within the globe. In general, only a very small percentage of the administered dose of drug, present in the precorneal area, crosses the cornea and arrives in the intraocular tissues. The rest of the administered drug is dragged by the tears that bathe the anterior face of the ocular globe to the nasal conduit, where it is systemically absorbed or later eliminated by the digestive tract. The limited time of contact of the drug with the cornea leads, in many cases, to an insufficient dose of medicine reaching intraocular tissues. For this reason, it is essential to have a "device" that can measure this transcorneal penetration.

In recent years several studies have been carried out to estimate ocular bioavailability "ex vivo" on the cornea using corneal perfusion chambers. There are corneal chambers that only serve as a simple diffusion system to compare formulations [1–3], or there are corneal perfusion chambers that try to reproduce as best as

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possible the physiological conditions, measuring the transcorneal potential to control the vitality of the cornea [4,5] or simulating tear flow in the external compartment and having air pressure in the internal compartment [6,7]. Some of the most complete systems do not measure transcorneal potential, which we consider a crucial aspect.

Besides, evaluation of some of the previously reported results is often difficult, since the instrument used to collect data is not always fully described or validated.

The main goal of this study was the validation, according to the International Conference on Harmonization Guidances [8,9], of a corneal perfusion system, designed and developed in our laboratory to determine drug permeation through the rabbit eye cornea. Sodium fluorescein was chosen as a model for "ex vivo" permeability studies as it is widely used in medicine, it may be considered safe and it is suitable for fluorescence techniques.

The transcorneal perfusion of the non-steroidal anti-inflammatory drug (NSAID) sodium diclofenac was assayed in direct solution in artificial tears and coupled with β-cyclodextrin to determine the capacity of this additive to enhance corneal transport.

The corneal transport of sodium diclofenac solutions in artificial tears was also compared to other commercial sodium diclofenac eye drops in order to examine the effects of the additives on corneal drug permeation.

2. Materials and methods

2.1. Isolation of living rabbit corneas

For "ex vivo" corneal transport assessments, adult albino New Zealand rabbits weighing 1.8–2.2 kg, with no particular pre-treatment regimen were used as the animal model. Each experiment was performed with a group of six rabbits. A control cornea, without dye or drug, was used as the zero (white value) for the fluorescence spectroscopy analysis (FSA) or for ultraviolet absorption spectroscopy in each experimental procedure. Following ethics committee requirements, the rabbits were first anesthetized and then killed with an overdose of Nembutal injected through the marginal ear veins. Corneas free of defects were removed by dissection along with a 2–3 mm surround of sclera to assist in subsequent handling, with care taken to avoid damage to the corneal epithelium and endothelium. The reason for leaving some sclera attached was to prevent the epithelial or endothelial tissues of the cornea coming into contact with the chamber. The cornea was then immediately clamped in the chamber. A total of six rabbits (12 corneas) were used for each drug dose.

2.2. Artificial tears and aqueous humor

The artificial tear solution (ATS) used on the epithelial side of the cornea, was a bicarbonate buffered Ringer's solution (NaCl 6.52 g L^{-1}; KCl 0.36 g L^{-1}; Ca $Cl_2{\cdot}2H_2O$ 0.15 g L^{-1}; $MgCl_2{\cdot}2H_2O$ 0.16 g L^{-1}; $NaH_2PO_4{\cdot}2H_2O$ 0.12 g L^{-1}; $NaHCO_3$ 2.45 g L^{-1}) prepared previously and maintained at 4 °C. Prior to performing the experiment the above solution was warmed and 0.9 g L^{-1} of glucose was added and stirred.

To prepare the artificial aqueous humor (AAH) for the endothelial side of the cornea, glutathione 0.13 g L^{-1} and adenosine 0.09 g L^{-1} at 37 °C were added to the ATS just before use. All solutions were prepared from reagent grade chemicals as in the report of O'Brien and Edelhauser [10].

2.3. Fluorescein solution preparation

Solutions containing from 4 to 14 mg mL^{-1} of fluorescein sodium salt (Sigma F6377), in ATS were prepared with magnetic stirring.

2.4. Sodium diclofenac solution preparation

Solutions containing 0.5 mg mL^{-1} or 1 mg mL^{-1} of sodium diclofenac (Sigma D6899) in ATS were prepared with magnetic stirring.

2.5. Diclofenac/β-cyclodextrin complex formation

To obtain the complex, 376 mg of sodium diclofenac and 1135 mg of β-cyclodextrin (Sigma C4767) were mixed in a mortar. Then a few drops of water were added, followed by kneading for 15–30 min to obtain a uniform paste. The resulting dough was poured onto a metallic surface and dried in an oven at 40 °C for 24 h. The dried product was crushed in a mortar and sieved to obtain a fine powder. The concentration of the diclofenac in the complex was determined by HPLC chromatography.

Solutions of the diclofenac/β-cyclodextrin complex containing 0.1 mg mL^{-1} of diclofenac in ATS were obtained.

2.6. Commercial eye drops used

To compare the corneal penetration of the commercial eye drops containing diclofenac with the diclofenac-free solution and the β-cyclodentrin complex, two commercial drugs were used:

- *Diclofenaco Lepori® eye drops*: Sodium diclofenac 0.1% solution in water for injection, with L-lysine monohydrate, sodium borate, sodium chloride, polyoxyethylenated ricin oil, benzalkonium chloride and sodium edetate.
- *Voltaren® eye drops*: Sodium diclofenac in the form of a hydroxypropyl-γ-cyclodextrin complex 0.1% solution, with hydrochloric acid, benzalkonium chloride, sodium edetate, propylenglycol, tromethamol and tyloxapol.

2.7. The transcorneal apparatus

Fig. 1 shows the entire setup of the system. The device was designed and constructed in our laboratory based on the devices described by Richman and Tang-Liu [6] and Thiel et al. [7]. The principle of the apparatus is that the cornea of the rabbit is placed in a methacrylate chamber clamped between two pieces so that the epithelial surface faces one compartment (anterior or tear compartment) and the endothelial surface the other (posterior or aqueous humor chamber). The fluid contained in the anterior compartment can be exchanged continuously by means of a pump. The hydrostatic pressure in the endothelial compartment can be chosen arbitrarily and independently. The experimental temperature can be controlled continuously. The transcorneal electrical potential can be continuously measured and recorded to check the vitality of the rabbit cornea.

The cornea fixing system consists of two identical methacrylate chambers (to simultaneously measure the two corneas of the same rabbit), each one set up as three pieces which can be assembled by means of two screws so that the cornea is clamped in a borehole of 9 mm diameter, leaving a 0.55 cm^2 corneal surface exposed. The dimensions of the chambers are chosen with a view to clamping rabbit cornea. The main piece (Fig. 2a) is a 70 mm × 40 mm × 60 mm methacrylate block which contains a cylindrical compartment of 7 mL volume to hold the AAH with a cork to avoid the loss of hydrostatic pressure. There is also a bore to introduce an electrode for the

measurement of transcorneal potential and a water circulation system to maintain the block at constant temperature. The other two items (Fig. 2b) are small methacrylate pieces which allow the chamber to be closed and the ATS with the dissolved drug to flow at the required flow rate, and also a borehole for the epithelial potential electrode. By means of two locking screws the pieces of the chamber are kept in a rigid position in relation to each other, so that there is constant compression of the cornea in the fixation zone.

The flow system for the external solution can be used to simulate the shed tears system. From a reservoir, the ATS fluid containing the dissolved drug is pumped by means of a tube pump (Miniplus 2-GILSON) at 2.0 mL/min (according to tear kinetics described by Maurice [11]) to the bottom of the above-mentioned methacrylate chambers. The fluid leaves the chamber by a hole in the top. The tube that leads the ATS to the chamber crosses a 3 way-connection for injecting drugs into the chamber using a syringe. The intake to the vials from the chambers takes place through a stainless steel tube.

The CO_2 and O_2 supply, necessary for the correct survival of the cornea, is provided by injecting carbogen (95% O_2, 5% CO_2) from a pressured bottle (Fig. 1), into the posterior part of the endothelial compartment through an existing orifice in the body of the chamber. The endothelial compartment is covered with a perforated rubber cork which contains a polyethylene tube that finishes in a water manometer accurate to within 1 mm of water. The depth of the outlet tube under the water surface determines the pressure in the chamber. By varying the height of the outlet tube the pressure in the chambers can be varied. In initial experiments, the carbogen injected into the internal chamber has to overcome the existing pressure in the end of the glass tube, which is submerged to a depth of 176.8 mm (13 mm of Hg), to create an equivalent pressure inside the chamber. The glass column must release a bubble once in a while to confirm that it is overcoming this pressure. A security system also exists so that if the inlet pressure of carbogen gas surpasses 280 mm of water (19 mm Hg), it bubbles and reduces pressure below that value. This way, the pressure inside the chamber will stay between 13 and 19 mm Hg [6].

To check the functional state of the cornea the potential across the cornea is measured through boreholes close to the cornea by means of two agar-gel bridges in polyethylene tubes (1.5 mm internal diameter, 1% agar in 3 M KCl). The agar bridges lead to a cartridge filled with agar screwed to Ag/AgCl voltage electrodes supplied by WPI (EKV Using electrode Kit). The potential difference between these two Ag/AgCl electrodes is recorded directly on a multimeter with a 50 Hz filter [12] supplied by Irisna. The corneal potential data were continuously plotted by a computer system to control corneal vitality. The computer system consists of a Data Acquisition Card, supplied by National Instruments, and software specifically adapted in our laboratory from the commercial software Labview.

The two-perfusion chambers have a hole through which warm water circulates from an external thermostatic bath system Selecta Tectron S-473·100 (Fig. 1). The temperature in the bath and in the methacrylate chambers can be read directly from a mercury thermometer. During the experiment the temperature in the chambers was maintained at 35 °C.

The agitation system is a magnetic stirrer SBS A-163 B. It contributes, together with the oxygenation system, to a homogeneous inner solution.

2.8. Sampling

The extraction of small volumes of the inner solution without loss of pressure was carried out by means of a syringe with a needle that crosses the cork of the inner compartment, thus preventing loss of pressure in the compartment. When the piston of the syringe is extracted, initial suction begins, and aided by the pressure of the leftover gas, the solution contained in the compartment flows slowly into the interior of the syringe. The required volume can

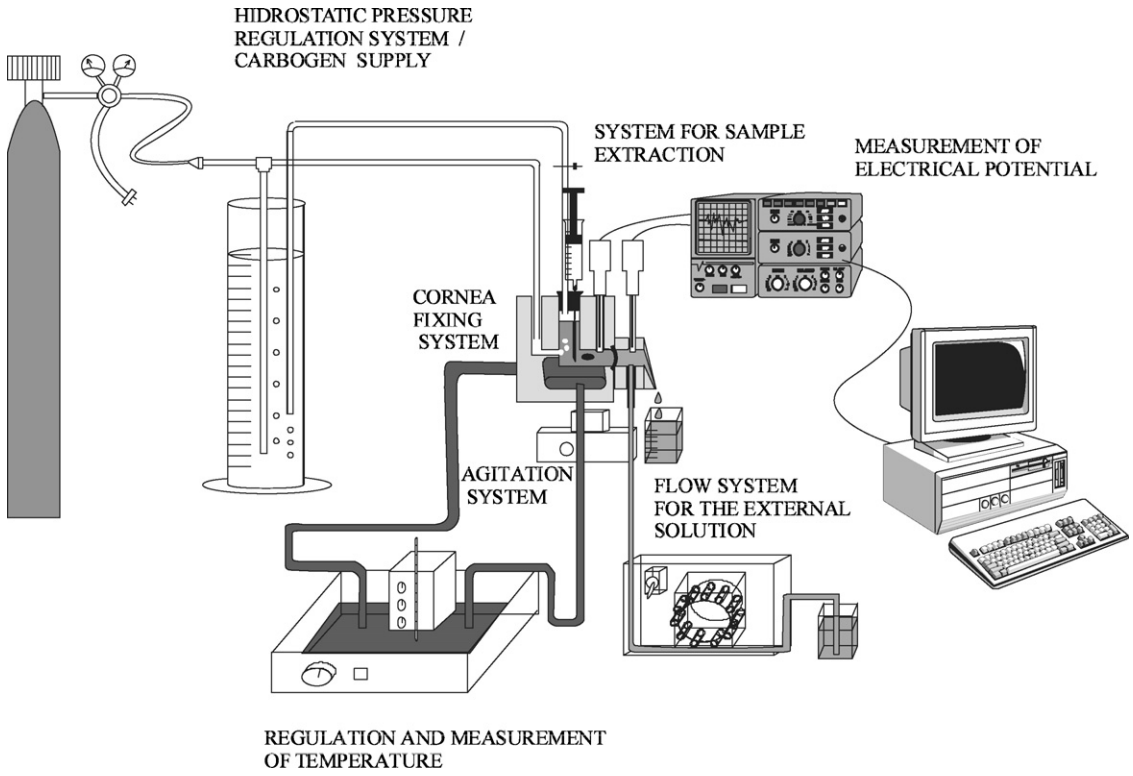

Fig. 1. General plot of the apparatus.

be extracted and then the piston is replaced, so the solution is introduced back into the compartment.

Samples of 50 μl of the endothelial compartment were taken at different times (between 5 min and 2 h) and then were analysed by fluorescence spectroscopy (fluorescein) or ultraviolet absorption spectroscopy (diclofenac). The endothelial compartment volume was maintained by addition of an equal volume of AAH when the samples were removed. Samples were diluted with AAH to obtain a final volume of 1 mL.

2.9. Analytical procedure

A spectrofluorometer Hitachi F-200 was used to measure fluorescein concentration. The instrument calibration was performed with fluorescein solutions in the range of 10^{-8} to 10^{-5} mg mL^{-1}. The fluorescence excitation/emission wavelengths were λ_{ex} = 487 and λ_{em} = 513 nm, respectively. No quenching due to artificial tears or aqueous humor was observed in repeated measures.

To determine the sodium diclofenac concentrations an ultraviolet absorption spectrophotometer Shimatzu UV-2401PC, adjusted to λ = 290 nm, was used. The concentration of diclofenac forming a complex with β-cyclodextrin was measured in a Waters 717 plus Autosampler HPLC Chromatograph with Hypersil ODS (5 mm, 100 cm × 4.6 cm) column and acetonitryl 25:methanol 25:sodium acetate (0.02 M, pH 7.0) 50 mobile phase. The UV detector was adjusted to λ = 290 nm.

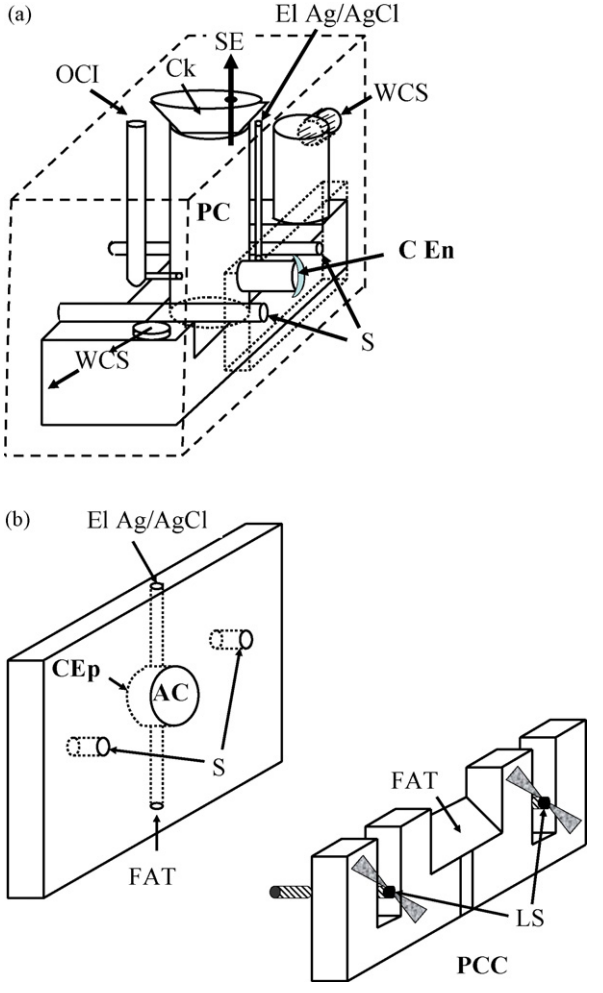

Fig. 2. Corneal perfusion chamber. (a) The main piece. (b) The other two pieces. PC: posterior, endothelial or aqueous humor compartment; AC: anterior, epithelial or tear compartment; CEn: cornea, endothelial side; CEp: cornea, epithelial side; Ck: cork; SE: sample extraction; S: screws; OCI: orifice to carbogen injecting (hydrostatic pressure control); WCS: water circulation system to temperature control; EAg/AgCl: electrode Ag/AgCl to measure the corneal potential; FAT: flux of artificial tear from a pump; PPC: piece to close the anterior compartment and clamp the cornea; LS: locking screws.

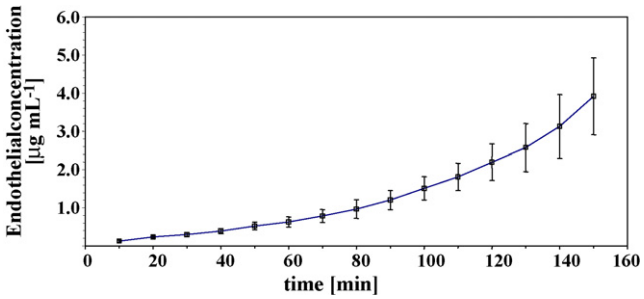

Fig. 3. Appearance of sodium fluorescein in the endothelial compartment of the corneal chamber (mean values and standard deviation) after perfusion in the epithelium side of a 5 mg mL^{-1} sodium fluorescein solution.

2.10. Statistical treatment

Using the fluorescence spectroscopy data, linearity and precision (repeatability, intermediate precision and robustness) of transcorneal permeability values were determined with SPSS software for Windows System.

2.11. Determination of apparent corneal permeability coefficients

Apparent permeability coefficients (P_{app}) were measured by previously described methods [13]:

$$P_{app} = \frac{\delta Q}{\delta t\, 60 A C_0} \quad (1)$$

where $\delta Q/\delta t$ is the permeation rate of drug across the cornea obtained from the slop of the linear portion of the graphic of corneal permebility versus time, 60 is the conversion from minutes to seconds, A is the corneal surface (in this study 0.55 cm^2) and C_0 the initial fluorescein or diclofenac concentration in the epithelial compartment.

3. Results

3.1. Transcorneal permeation

Fig. 3 plots the mean sodium fluorescein concentration in the inner (endothelial) compartment of the cornea against time (mean of 18 experiments), measured by fluorescence spectroscopy as previously described. The apparent corneal permeability coefficient for sodium fluorescein obtained from (1) is 3.82 × 10^{-4} cm/h.

3.2. Linearity

The resulting mean concentrations in the endothelial compartment of the corneal chamber at 150 min after continuous perfusion of six different concentrations of sodium fluorescein solutions into the epithelial side are plotted in Fig. 4. The coefficient r^2 calculated from the obtained regression line was 0.9918.

3.3. Precision (repeatability)

The resulting concentrations in the endothelial compartment of the corneal chamber were measured at 150 min after continuous perfusion of 10 mg mL^{-1} sodium fluorescein solution into

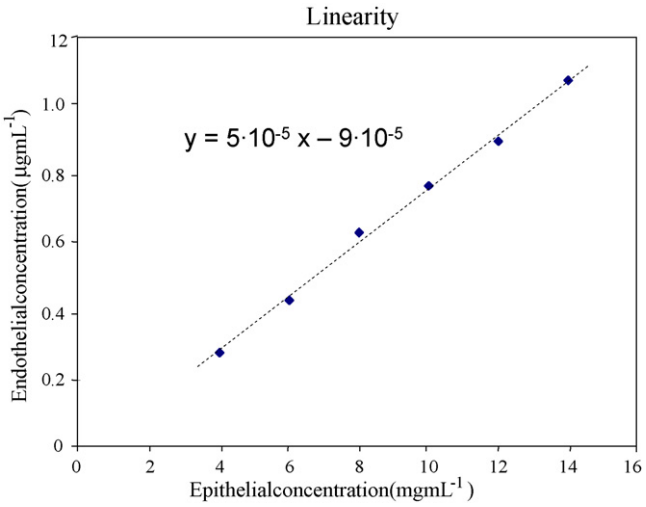

Fig. 4. Mean concentration and regression line of sodium fluorescein in the endothelial compartment after perfusion in the epithelial side of six different concentrations of sodium fluorescein.

the epithelial side for 12 different experiments performed by the same operator in identical experimental conditions. The mean concentration was 7.67×10^{-4} mg mL^{-1} and standard deviation (S.D.) 1.43×10^{-4} mg mL^{-1}

3.4. Intermediate precision

The consistency between individual results obtained during the normal and correct operation of the apparatus using identical test material, but with different operators, was also evaluated. Three different operators performed a series of six assays at different times (1-month intervals). The mean results are shown in Fig. 5.

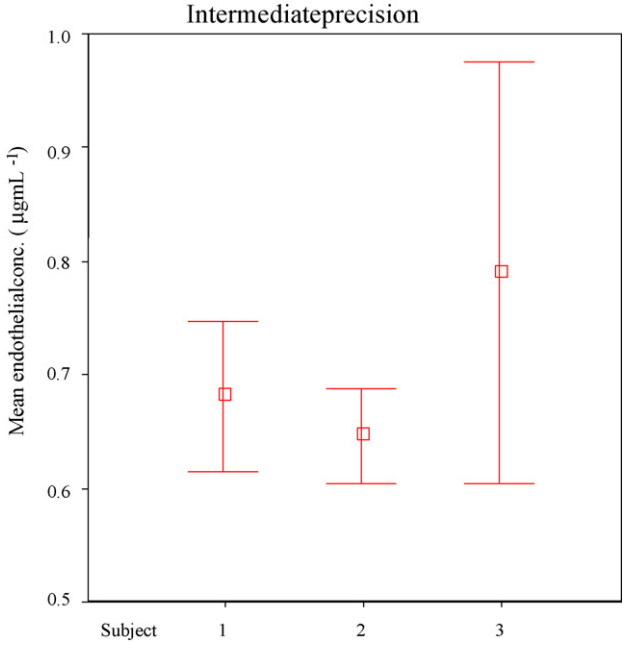

Fig. 5. Mean and 95% confidence interval (six assays) sodium fluorescein concentration (μg mL^{-1}) in endothelial compartment of the corneal chamber for different subjects determined after 150 min of perfusion of a 10 mg mL^{-1} solution in the epithelium side in 15 rabbit cornea.

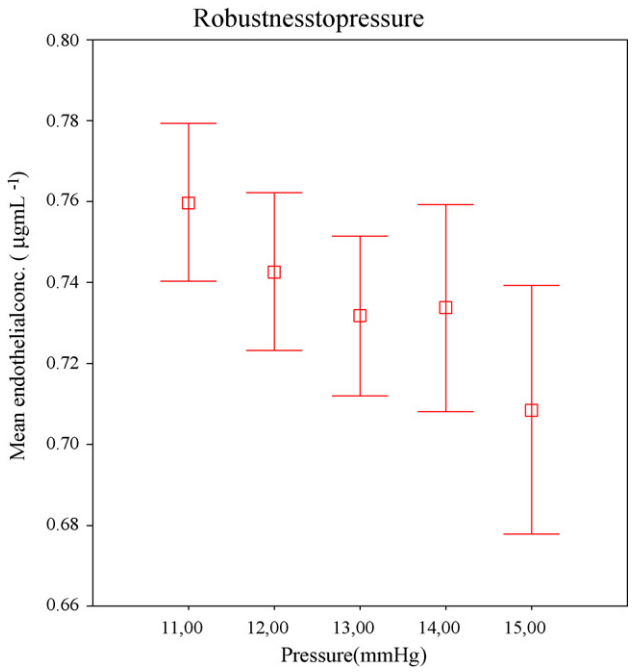

Fig. 6. Mean and 95% confidence interval (six assays) sodium fluorescein concentration (μg mL^{-1}) in endothelial compartment of the corneal chamber for different gas pressure determined after 120 min of perfusion of a 10 mg mL^{-1} solution in the epithelium side in six rabbit cornea.

3.5. Robustness

Three parameters are suitable for evaluating robustness of the device: gas (carbogen) pressure, bath temperature and corneal potential. Fig. 6 shows the influence of five different carbogen pressures on the concentration measured (six assays each). The effect of variable bath temperature on robustness is shown in Fig. 7. Table 1 and Fig. 8 show the effect on inner concentration when the corneal potential falls.

3.6. Corneal permeation of different formulations of sodium diclofenac

Fig. 9 shows the variable corneal permeation of sodium diclofenac solution in artificial tears and of the diclofenac/β-cyclodextrin complex.

Fig. 10 compares the corneal transport of the same dissolution of diclofenac in artificial tears with the commercial eye drops Diclofenaco-Lepori® and Voltaren®.

4. Discussion

The principal aim of the proposed device is to make available a useful tool to systematically study different formulations of the same drug, and to compare the effect of the formulation and additives in different pharmaceutical dosage forms. Furthermore, the

Table 1
Mean and standard deviation (S.D.) of sodium fluorescein concentration (mg mL^{-1}) in the endothelial compartment of the corneal chamber for different corneal potentials (<10 and >10 mV) determined after 60 min of perfusion of a 10 mg mL^{-1} solution into the epithelial side of 30 rabbit corneas

Corneal potential (mV)	No. of corneas	Mean ± S.D.
>10	24	$7.51 \times 10^{-4} \pm 2.53 \times 10^{-5}$
<10	6	$8.42 \times 10^{-4} \pm 7.10 \times 10^{-5}$

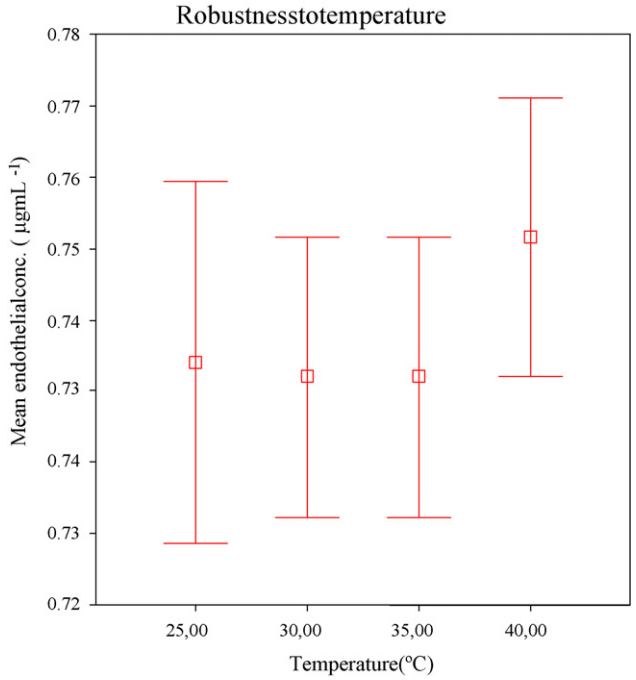

Fig. 7. Mean and 95% confidence interval (six assays) sodium fluorescein concentration ($\mu g\,mL^{-1}$) in endothelial compartment of the corneal chamber for different bath temperature determined after 120 min of perfusion of a 10 mg mL^{-1} solution in the epithelium side in six rabbit cornea.

use of an "ex vivo" method is commendable because it saves a great number of animal sacrifices, and reduces variability.

Validation of the corneal apparatus was carried out with fluorescein because this dye is widely used and is easy to measure at low concentrations. Fluorescein was also chosen because it penetrates slowly through the corneal tissues. The calculated corneal

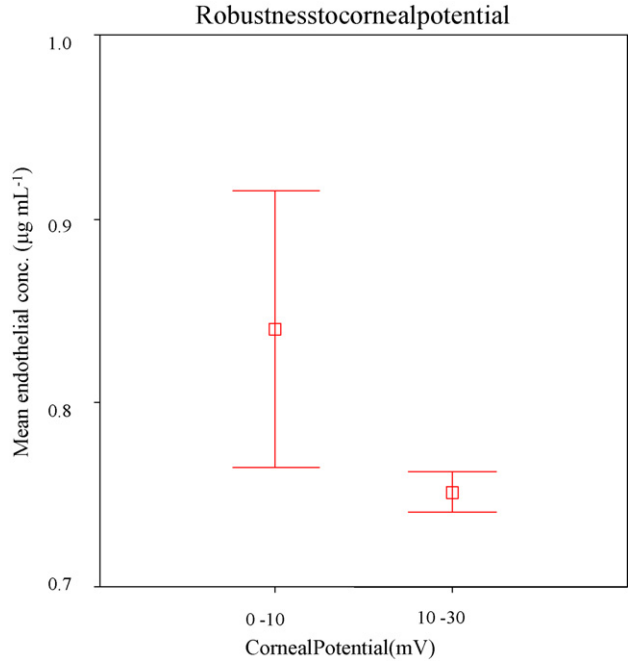

Fig. 8. Mean and 95% confidence interval (six assays) sodium fluorescein concentration ($\mu g\,mL^{-1}$) in endothelial compartment of the corneal chamber for different corneal potential determined after 60 min of perfusion of a 10 mg mL^{-1} solution in the epithelium side in 30 rabbit cornea clustered as <10 and <10 mV.

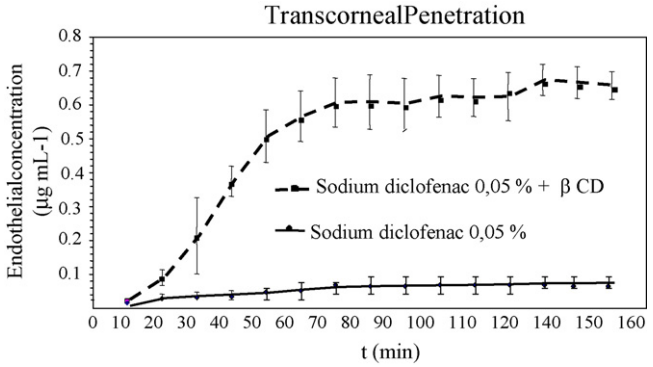

Fig. 9. Comparison of transcorneal permeation between sodium diclofenac 0.5 mg mL^{-1} solution and sodium diclofenac forming complex with β-cyclodextrin at the same concentration.

permeability is consistent with the values described in reference textbooks [14]. Ex vivo corneal permeability, however, does not predict in vivo bioavailability, because the latter is determined as the fraction of the dose reaching the aqueous humor. This is mostly dependent on the precorneal drainage rate. For this reason, the corneal apparatus described above has a flow changing system to modify the rate of drainage tears depending on the drug.

Measurements of the electric potential across the corneal preparation in all experiments showed that the corneal endothelium was always positive against the corneal epithelium. Generally, potential was maintained with small fluctuations or a slow fall-off for three to four hours. The scattergram shows that the range of peak potential differences reached in most experiments at 35 °C was from 15 to 42 mV. The potential values in the fluorescein experiments were lower than in the controls, as reported by Akiyama et al. [15]. The stability of cornea potential confirms that the integrity of the cornea is maintained during mounting and the experimental procedures. Experiments were discarded when this potential dropped below 10 mV, because in our robustness experiments we observed different results below this potential. For this reason, 10% of measurements were discarded.

The experimental measurements were not significantly affected by the gas (carbogen) pressure in the chamber in the interval tested (11–15 mm Hg). One-way Anova between groups showed only group differences at a significance level of 0.100 (*F*-test).

We assumed that temperature increases would lead to higher corneal permeation, and therefore that temperature must adjusted and controlled during measurements. However, one-way Anova

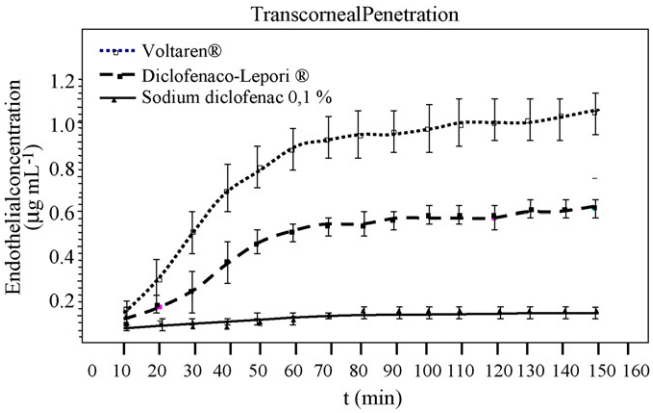

Fig. 10. Comparision of transcorneal permeation among sodium diclofenac 1 mg mL^{-1} solution, Diclofenac-Lepori® and Voltaren® eye drops.

between groups showed group differences at a significance level of 0.291 (*F*-test), suggesting that there are no significant differences at temperatures between 25 and 40 °C.

Finally electrical potential was an important parameter related to cornea vitality, and must be accurately controlled. Below 10 mV corneal permeability increases significantly. A significance value for the t test of 0.029 indicates that there is a significant difference between the two 0–10 and 10–30 mV groups.

The results obtained from comparing corneal permeation of sodium diclofenac direct 0.5 mg mL^{-1} solution in artificial tears with Diclofenac/β-cyclodextrin complex (Fig. 9) show a significant increase of corneal permeation with the latter. The calculated apparent corneal permeability coefficient of diclofenac is doubled when diclofenac forms a complex with β-cyclodextrin.

As Fig. 10 shows, a good formulation can increase diclofenac corneal permeation up to ten times. However if cyclodextrins are included in the formulation (Voltaren®), corneal transport can increase even more.

5. Conclusions

The model of permeability diffusion used in this work is a good approach for comparing the corneal transport of different formulations of the same drug.

In all the experimental conditions the fluorescein concentration in the endothelial compartment increased following an exponential plot over time, with a good correlation between the epithelial and endothelial concentration.

The system is robust against slight modifications of temperature, and is also quite robust against slight changes in hydrostatic pressure, although it is essential to control the corneal potential to avoid important measurement errors.

The proposed device shows good precision when measuring transport through rabbit cornea at different drug concentrations, and good repeatability and intermediate precision with non-significant differences among operators in repeated experiments. For this reason the proposed device is a valuable instrument for the measurement of corneal permeation of drugs applied topically into the eye.

Finally the method employed shows how different formulations of the same drug permeate in variable amounts depending on the additives used in the formulation.

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