Development of a Convenient *ex vivo* Model for the Study of the Transcorneal Permeation of Drugs: Histological and Permeability Evaluation

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ABSTRACT: In this paper, an *ex vivo* model for the study of the transcorneal permeation of drugs, based on porcine tissues, was evaluated. The setup is characterized by ease of realization, absence of O_2 and CO_2 bubbling and low cost; additionally, the large availability of porcine tissue permits a high throughput. Histological images showed the comparability between porcine and human corneas and confirmed the effectiveness of the isolation procedure. A new de-epithelization procedure based on a thermal approach was also set up to simulate cornea permeability in pathological conditions. The procedure did not affect the integrity of the underlying layers and allowed the characterization of the barrier properties of epithelium and stroma. Six compounds with different physicochemical properties were tested: fluorescein, atenolol, propranolol, diclofenac, ganciclovir and lidocaine. The model highlighted the barrier function played by epithelium toward the diffusion of hydrophilic compounds and the permselectivity with regard to more lipophilic molecules. In particular, positively charged compounds showed a significantly higher transcorneal permeability than negatively charged compounds. The comparability of results with literature data supports the goodness and the robustness of the model, especially taking into account the behavior of fluorescein, which is generally considered a marker of tissue integrity. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:63–71, 2015

Keywords: *in vitro* model; permeability; drug transport; epithelial delivery/permeability; mucosal drug delivery; transcorneal; permselectivity; porcine cornea; ocular delivery

INTRODUCTION

Several diseases affect the anterior segment of the eye and topical formulations, such as eye-drops, ointments and gels, represent the simplest and most used treatment approach. However, part of the drug applied topically on the ocular surface is lost due to tear turnover, binding to tear proteins, non-corneal absorption through sclera and conjunctiva, and systemic absorption.[1] Additionally, the cornea, because of its complex structure, exerts a high resistance toward xenobiotic permeation. All these phenomena preclude the possibility of a consistent transcorneal permeation: it has been estimated that approximately 7% of lipophilic and 1% of hydrophilic compounds applied topically could reach the aqueous humor.[2] Furthermore, the cornea can also represent the target of different pathological conditions, such as keratitis—that can evolve to ulcer—neovascularization, keratoconus and cystinosis.

Different models are currently used to study the transcorneal permeability, both *in vivo* and *in vitro*/*ex vivo*. *In vivo* models are mainly based on rodents (rabbit, mouse, rat), whereas *in vitro*/*ex vivo* models include epithelial cells layer cultures,[3] reconstructed cornea[4] or excised cornea; the latter reproduces the complexity of the whole membrane and allows to investigate the transport phenomena. *In vitro* models are characterized by a good control of experimental conditions and are relatively cheap, although they allow to study only a single step in the absorption process.

Porcine eyes are a good model of human eyes,[5] are easily available and the cornea is simple to isolate and adequately large and robust to handle. This model has already been used in the literature, however, it is poorly characterized in terms of histology and experimental setup.

The aim of the present paper was to develop a simple and robust *ex vivo* model for the transcorneal permeation of drugs, based on freshly explanted porcine cornea. The model is easy to set up, cheap, and can give results comparable to other methods reported in the literature, but characterized by more complex setup such as O_2 and CO_2 bubbling, nutrients addition and special diffusion cells. Furthermore, a new method for epithelium removal, based on a thermal approach and never reported before in the literature for the cornea treatment, was identified, in order to obtain a tissue simulating a condition of compromised epithelium. The full-thickness and de-epithelialized model was evaluated by histological analysis and permeation experiments on Franz-type diffusion cell. As model permeants six compounds of different physicochemical properties with diagnostic or therapeutic rationale were chosen: a dye [fluorescein (FLUO)], two beta-blockers [atenolol (ATNL) and propranolol (PRPNL)], a NSAID [diclofenac (DICL)], an antiviral [ganciclovir (GCV)] and a local anesthetic [lidocaine (LIDO)]. Together with the evaluation of the model, also interesting results on the role of the charge of compounds with similar size and lipophilicity on transcorneal permeation are reported.

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MATERIALS AND METHODS

Materials

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) as well as atenolol, diclofenac sodium, fluorescein sodium, ganciclovir and propranolol as hydrochloride were purchased from Sigma–Aldrich (St. Louis, Missouri, USA); LIDO as hydrochloride was a gift from Lisapharma S.p.A. (Como, Italy). Buffered solutions used were HEPES-buffered saline (HEPES; 5.96 g/L HEPES, 9.0 g/L NaCl pH 7.4 with NaOH 5 N) and phosphate-buffered saline (PBS; 0.19 g/L KH_2PO_4, 5.98 g/L $Na_2HPO_4 \cdot 12H_2O$, 8.8 g/L NaCl pH 7.4 with H_3PO_4).

For HPLC analysis, methanol, acetonitrile (both HPLC grade) and distilled water were used. All other chemicals used were of analytical grade.

Methods

Tissue Preparation

Fresh porcine eyes were isolated from Landrace and Large White pigs (age 10–11 months, weight 145–190 kg, both female and male animals), supplied from a local slaughterhouse (Annoni S.p.A., Parma, Italy). The eyes were kept in PBS at +4°C until use, which occurred within 2 h from enucleation. Only bulbs with macroscopically intact corneas were employed, whereas eyes showing opaque corneas were discarded. The full-thickness corneas (consisting of epithelium, stroma and endothelium) were isolated as corneo-scleral button by cutting with a scalpel beyond the limbus. To obtain de-epithelialized cornea samples, the whole bulb was soaked in 60°C deionized water for 2 min (the epithelium becomes completely opaque). Then, the epithelium was carefully peeled off using a spatula and discarded. To avoid tissue damage during the preparation procedure, the corneal tissues were exposed to air only for few minutes altogether; once prepared, all samples, both full-thickness and de-epithelialized, were kept in saline solution until use, that occurred within 30 min.

Tissue Characterization

Porcine cornea was characterized in terms of thickness and water content. The thickness was measured by means of a digital calliper (0.001 mm resolution; Absolute Digimatic 547–401; Mitutoyo, Milan, Italy): corneo-scleral buttons were measured at the central and peripheral (limbial) sections. Furthermore, the thickness of each of the five layers constituting the cornea was derived from the histological images.

For the determination of corneal water content, tissue discs of 9 mm diameter were cut, weighted and then dried at room temperature to constant weight in a desiccator containing calcium chloride. The water content (%) was calculated as follow:

$$\text{Water content}\,(\%) = ((w_\text{i} - w_\text{f})/w_\text{i}) * 100 \qquad (1)$$

where w_i and w_f are the initial and the final weight, respectively.

For optical microscopy, corneal samples, both full-thickness and de-epithelialized, just isolated or after permeation experiments, were fixed in 10% formaldehyde, then embedded in paraffin and sectioned using a microtome. Staining was carried out using Harris hematoxylin/eosin.[6] Images were taken using an optical microscope Nikon Eclipse 80i, equipped with a camera Nikon Digital Sight DS-2Mv and connected to the control software, NIS Elements F (Nikon Instruments, Calenzano, Italy).

Furthermore, porcine eyes were weighed and anatomical axis length was measured; aqueous and vitreous volumes were determined by weighting, assuming that density was 1 g/cm^3.

Permeation Experiments

Permeation experiments were performed in glass Franz-type diffusion cells (area 0.2 cm^2). Full-thickness or de-epithelialized tissues were placed on the cell with the endothelial side facing the receiving compartment.

The donor compartment was filled with 300 µL of solution containing the model compound, dissolved in HEPES buffer at different concentrations (ATNL 2 mg/mL, DICL 1 mg/mL, FLUO 1 mg/mL, GCV 1.5 mg/mL, LIDO 2 mg/mL and PRPNL 0.1 mg/mL). The concentrations have been selected based on the data found in the literature in analogous experiments, for ease of comparison with previously published data. In case of DICL and GCV, these concentrations also match the therapeutic ones. The receiving phase consisted of 4 mL of HEPES buffer, thermostated at 37°C and magnetically stirred to avoid any boundary layer effect. At predetermined times for up to 5 h, 300 µL of solution was sampled from the endothelial side and immediately replaced by an equal volume of fresh HEPES buffer.

Blank experiments were conducted to exclude the presence of any interference from the tissue; all experiments were carried out at least in triplicate using always different ocular bulbs from different animals.

Analytical Methods

FLUO was analyzed without any preliminary separation by fluorescence (excitation wavelength 485 nm, emission wavelength 535 nm) with multilabel plate reader (Viktor^3 1420; Wallac, PerkinElmer, Waltham, MA, USA). The calibration curve was built with standard solutions between 5 and 250 ng/mL.

ATNL, DICL, GCV, LIDO, and PRPNL were analyzed using an HPLC apparatus equipped with an isocratic pump (Series 200, PerkinElmer), an autosampler (Prostar 410; Varian, Leinì (To), Italy) and a UV–Vis spectrophotometric detector (LC290 PerkinElmer or SPD-20ALC Shimadzu, Kyoto, Japan) or, as an alternative, by HPLC/UV–Vis Flexar (PerkinElmer). The analyses were performed at room temperature, unless otherwise indicated. All methods were validated for linearity, accuracy (RE%) and precision (RSD%). Chromatographic conditions are described in detail in Table 1.

Data Processing

Data were presented as amount permeated (µg/cm^2) as a function of time (min). The transcorneal flux across full-thickness and de-epithelialized cornea (J, µg/cm^{2}min) was calculated as the slope of the regression line at steady state, whereas the apparent permeability coefficient of full-thickness and de-epithelialized cornea (P_app, cm/s) was calculated at the steady state as:

$$P_\text{app} = J/C_\text{D} \qquad (2)$$

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