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# Human cornea construct HCC—an alternative for in vitro permeation studies? A comparison with human donor corneas☆

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**Abstract**

Transcorneal in vitro permeation studies of ophthalmic drugs are normally performed with either excised animal corneas or latterly corneal cell culture models. A good correlation between these models and excised animal corneas regarding permeation behaviour of drugs has already been shown. However, comparisons between corneal in vitro models containing human cells and excised human corneas do not exist yet. Therefore in the present study the transcorneal permeation of six different model drugs (pilocarpine hydrochloride, befunolol hydrochloride, hydrocortisone, diclofenac sodium, clindamycin hydrochloride and timolol maleate) across our previously described three-dimensional organotypic human cornea construct (HCC) was tested using Franz diffusion cells and compared with permeation data obtained from human donor corneas. The HCC showed a similar permeation behaviour compared with human donor cornea for all substances. The permeabilities (permeation coefficients *P*) of the human cornea equivalent versus the human donor cornea were the same in the case of diclofenac, clindamycin, timolol, but marginally decreased for hydrocortisone and slightly increased for pilocarpine and befunolol. These small differences of permeation coefficients were expressed as factors and only varied from 0.8 to 1.4. The results indicate that the HCC may be an alternative for in vitro permeation studies and appropriate for predicting drug absorption into the human eye.


*Keywords:* Human cornea equivalent; Cell culture; In vitro model; Drug permeation studies; Human donor cornea

## 1. Introduction

Since the cornea is the main barrier for the transport of substances into the eye, the extent of permeation of topically applied ophthalmic drugs across the cornea is an important biopharmaceutical parameter. Therefore, there is a great need of transcorneal permeation data in the discovery, development and selection of new ophthalmic drugs or drug delivery systems for application to the eye. For these purposes, in vitro experiments have to be performed. Such permeability studies usually use isolated corneal tissue mounted in modified side-by-side diffusion chambers. Excised corneas from slaughtered animals or laboratory animals are frequently used [1], in particular from rabbits, although anatomical and physiological differences between the rabbit and human eye suggest that in vitro permeation data could also differ [2].

In order to replace excised animal corneas, several in vitro models based on cell culture techniques have been proposed in the past decade and their usefulness for in vitro permeation has recently been shown [3–6]. But comparison was only made with data obtained from excised animal cornea so far, because in vitro permeation data of human cornea do not exist, except for only a few studies [7]. Hence in the present study, the degree of comparability between an organotypical human cornea construct (HCC) described before [8] and human donor cornea was investigated regarding permeability of a variety of typical ophthalmic drugs.

With regard to original (the cornea, a multilayered tissue, is made up of three major cellular layers, the epithelium,

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the stroma and the endothelium), the three-dimensional HCC was reconstructed step-by-step with epithelial cells cultivated on stromal cells embedded in a collagen matrix with an underlying layer of endothelial cells [8]. Pilocarpine hydrochloride (PHCl), befunolol hydrochloride (BHCl), timolol maleate (TM), hydrocortisone (HC), diclofenac sodium (DNa) and clindamycin hydrochloride (CHCl) were chosen as ophthalmic drug models because they are frequently used in the treatment of glaucoma, inflammatory and infectious diseases. Furthermore, they represent different physico-chemical properties (log partition coefficient octanol/water varied from −1.3 to 1.6 and molecular weight from 208 to 461 g/mol). Permeation data obtained with HCC were compared with those from human donor cornea.

## 2. Material and methods

### 2.1. Materials

Borocarpin® S 2% from Dr Winzer (Olching, Germany) containing 2% pilocarpine hydrochloride (0.01% benzalkonium chloride as preservative) and Glauconex® 0.5% from Alcon-Pharma (Freiburg, Germany) containing 0.5% befunolol hydrochloride (0.007% benzalkonium chloride) are commercial aqueous eye drop solutions (benzalkonium chloride, known as substance with permeation enhancing potential, influenced both cornea construct and excised cornea in the same manner [data not shown]). HC 0.02% is an aqueous solution of 0.02% hydrocortisone. Diclofenac sodium (DNa) 0.5%, timolol maleate (TM) 0.5% and clindamycin hydrochloride (CHCl) 0.5% are also used as aqueous solutions containing 0.5% (w/w) drug, respectively. Isotonic conditions were adjusted by addition of sodium chloride in all solutions. The pH values varied from pH 4.6 (timolol maleate) to 7.0 (Glauconex®), adjustment of pH with acids or bases was not carried out. Freshly prepared solutions were free of preservative.

### 2.2. Human cornea construct—HCC

Immortalised cell lines, i.e. SV 40 transfected human corneal epithelial (CEPI 17 CL 4) and endothelial cells (HENC), as well as primary isolated native human corneal fibroblast were used for reconstruction of the human cornea in vitro. Standard cultivation and characterization of cells have already been reported earlier [9,10]. Cornea equivalent was constructed step-by-step in Transwell® (Costar, Fern-wald, Germany) cell culture inserts as described by Reichl et al. [8]. Endothelial cells (HENC) were seeded onto a polycarbonate filter covered with a layer of type I collagen, acid-extracted from rat tail, and grown to confluence within 7 days in medium F99—a 1:1 medium mixture of Ham's F12 and Medium 199 (Gibco BRL Life Technologies, Karlsruhe, Germany) supplemented with 5% fetal calf serum (FCS; Biochrom KG, Berlin, Germany) and 1% antibiotic/antimycotic solution (Gibco BRL). A type I collagen gel matrix containing stromal fibroblasts was then cast atop the confluent endothelial cell layer. Endothelium stroma equivalent was cultivated approximately 4 days submerged in DMEM medium supplemented with 10% FCS, 4 mM L-glutamine and 1% antibiotic/antimycotic solution (PAA Laboratories, Linz, Austria). Epithelial cells (CEPI 17 CL 4) were seeded onto the contracted collagen lattice and grown in medium DMEM/F12—a 1:1 medium mixture of DMEM (ICN, Eschwege, Germany) and Ham's F12 (Gibco BRL) supplemented with 5% FCS, 5 μg/ml insulin, 1.4 μM hydrocortisone, 1 ng/ml epidermal growth factor (EGF; Biochrom), 10 μg/ml transferrin (Biofluids Biosource International, Nivelles, Belgium), 2 mM L-glutamine and 1% antibiotic/antimycotic solution (Gibco BRL)—for additional 7 days submerged to confluence. After epithelium became confluent tissue construct was lifted to the air–liquid interface for additional 10 days and cultivated in medium DMEM/F12 with reduced serum content of 2%. Within 10 days a multilayered epithelium was formed. The cultures were maintained in a humidified incubator at 37 °C with 5% $CO_2$ and medium was replaced three times per week.

### 2.3. Human donor cornea

Human donor corneas used in these experiments, which were deemed inappropriate for transplantation due to various reasons, e.g. low endothelial density (about 2.000 cells/mm$^2$) or infectious diseases (hepatitis B), were obtained from the Cornea Banks of Hannover and Hamburg (Germany) from 16 patients (mean age, 58 ± 19.6 SD; age range, 35–78 years). Corneas were cultured in Minimal Essential Medium MEM (Biochrom) supplemented with 2% FCS, penicillin (100,000 E/l), streptomycin (100 mg/l) and amphotericin B (2.5 mg/l) (Biochrom) [11] during organ storage for 12–21 days after enucleations. Prior and after permeation experiments, the epithelial appearance of the corneas was microscopically evaluated, there was no evidence of damages with the potential of influencing permeability characteristics (intact epithelial layers without degenerative indications were detected).

### 2.4. Permeation studies, calculation of flux values and permeation coefficients

Diffusion experiments were performed for 420 min using modified Franz diffusion cells at 37 °C to evaluate transcorneal drug permeability of six model drugs in aqueous solutions as described above across both human donor cornea and HCC. The receiver solution contained isotonic phosphate buffered saline (pH 7.4) and was stirred with a magnetic stirrer (Janke&Kunkel, Staufen, Germany) at 400 rpm during the experiment. Samples were taken from

the receiver chamber at fixed time intervals (every 60 min) and quantitatively analysed by HPLC.

The permeation parameters of model drugs were calculated by plotting the amounts of drug permeated through excised cornea or cornea construct (μg/cm$^2$) versus time (min). The steady-state flux ($J$) values across HCC or human cornea were evaluated from the linear ascents of the permeation graphs by means of the relationship

$$J = \frac{dQ}{dt\,A}(\mu g/(cm^2\ s))$$

where $Q$ indicates the quantity of substance crossing HCC or human cornea; $A$ the corneal area exposed; and $t$ the time of exposure. The permeation coefficient $P$ was calculated as

$$P = \frac{J}{c_o} \quad (cm/s)$$

where $c_o$ represents the initial drug concentration in the donor compartment.

### 2.5. HPLC methodology

Concentrations of model drugs in the samples were determined using a Waters 515, 717 plus, 486 HPLC system (Waters, Eschborn, Germany) at ambient temperature, using columns of Gromsil® 120 ODS-3 CP 5 μm, 125×4 mm (Grom, Herrenberg, Germany) in the case of pilocarpine hydrochloride and befunolol hydrochloride, of Hypersil® ODS 5 μm 125×4 mm (Grom) for diclofenac sodium and Hypersil® ODS 5 μm 250×4 mm for hydrocortisone, while a Symmetry Shield Reversed Phase (RP 8) column 150×3.9 mm (Waters) was used for timolol maleate and an EC 250×4.6 mm Nucleosil® 100-10 C18 column (Macherey–Nagel, Düren, Germany) for clindamycin hydrochloride. The standard HPLC methods used for the six substances have already been described earlier [8,12–14]. Data analysis and calculation were performed by Waters Millenium 32 Chromatography Manager software (Waters). The correlation coefficients of calibrations were at least 0.999 for all substances.

## 3. Results and discussion

The human cornea construct HCC had been cultivated as already reported and their epithelial compactness demonstrated as well. Both HCC and human cornea showed a multilayered epithelium. Hence a normal corneal permeation barrier was assumed. In order to investigate the usefulness of HCC for in vitro permeation studies, multiple permeation experiments were performed with six different commonly used model drugs across HCC and human donor corneas and the data, i.e. calculated permeation coefficients, were compared. The permeation profiles, i.e. permeated amount per unit area versus time, of all model drugs through HCC and excised human cornea are presented in Figs. 1 and 2, respectively. The figures clearly show that the extent of drug permeated into the receiver compartment was rather similar for HCC and human cornea. Furthermore, the resulting permeation coefficients $P$ were calculated and expressed as mean ± standard deviation and are shown in Table 1. The $P$ values varied from 5.4 to 14.6×10$^{-6}$ cm/s in the case of HCC and from 6.6 to 14.5×10$^{-6}$ cm/s for the human donor cornea. The lowest permeability with both HCC and human cornea was obtained for HC and the highest was observed in the case of DNa. Similar $P$ values were found for TM and BHCl as to approximately 8×10$^{-6}$ cm/s as well as for DNa and CHCl as to approximately 14×10$^{-6}$ cm/s. Furthermore, comparison of permeability between HCC and human donor cornea for each drug was expressed as a factor ($P$ value of

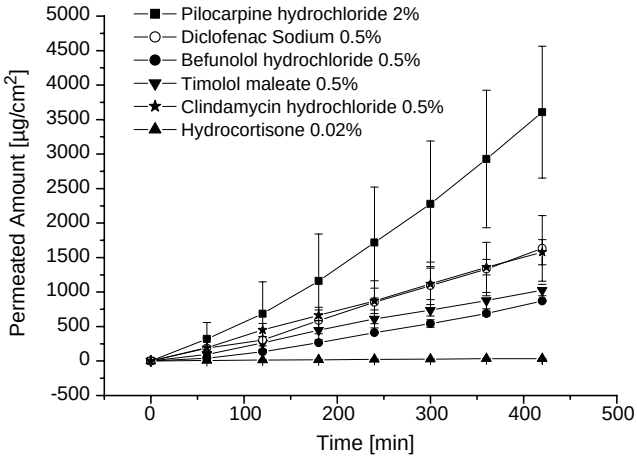


Fig. 1. Permeated amounts per unit area of pilocarpine hydrochloride (PHCl), diclofenac sodium (DNa), befunolol hydrochloride (BHCl), timolol maleate (TM), clindamycin hydrochloride (CHCl) and hydrocortisone (HC) versus time across human donor cornea. Each point represents the mean ± SD of three independent determinations.

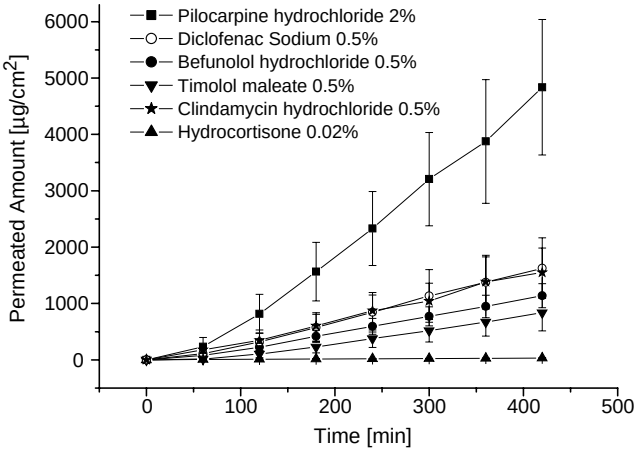


Fig. 2. Permeated amounts per unit area of pilocarpine hydrochloride (PHCl), diclofenac sodium (DNa), befunolol hydrochloride (BHCl), timolol maleate (TM), clindamycin hydrochloride (CHCl) and hydrocortisone (HC) versus time across human cornea construct (HCC). Each point represents the mean ± SD of 3–6 independent determinations.

Table 1
Drug permeability of HCC compared with human donor cornea

| Donor | Human donor cornea | Human cornea construct | Factor |
|---|---|---|---|
| Pilocarpine hydrochloride 2% | $9.98\times10^{-6}\pm1.03\times10^{-6}$ | $13.4\times10^{-6}\pm3.01\times10^{-6}$[a] | 1.3 |
| Diclofenac sodium 0.5% | $14.45\times10^{-6}\pm3.60\times10^{-6}$ | $14.63\times10^{-6}\pm4.21\times10^{-6}$ | 1.0 |
| Befunolol hydrochloride 0.5% | $7.05\times10^{-6}\pm1.11\times10^{-6}$ | $9.88\times10^{-6}\pm1.79\times10^{-6}$[a] | 1.4 |
| Timolol maleate 0.5% | $8.32\times10^{-6}\pm0.26\times10^{-6}$ | $8.13\times10^{-6}\pm2.93\times10^{-6}$ | 1.0 |
| Clindamycin hydrochloride 0.5% | $12.63\times10^{-6}\pm1.16\times10^{-6}$ | $13.47\times10^{-6}\pm3.87\times10^{-6}$ | 1.1 |
| Hydrocortisone 0.02% | $6.55\times10^{-6}\pm1.50\times10^{-6}$ | $5.41\times10^{-6}\pm0.40\times10^{-6}$[a] | 0.8 |

Permeation coefficients $P$ (cm/s) of pilocarpine hydrochloride (PHCl), diclofenac sodium (DNa), befunolol hydrochloride (BHCl), timolol maleate (TM), clindamycin hydrochloride (CHCl) and hydrocortisone (HC), mean ± SD ($n$ = 3–6). Differences between permeabilities of HCC and human donor cornea are expressed as factor.
[a] Ref. [8].

HCC divided by $P$ value of human cornea) and is shown in the right column of Table 1. The difference between HCC and human cornea varies by a fairly small factor from 0.8 to 1.4. Marginal differences were found in the case of PHCl, BHCl and HC while for DNa, TM and CHCl permeation coefficients were rather the same. In average the factor was just 1.1.

The results reveal a good correlation in permeability between HCC and human cornea. The averaged factor 1.1 from six examined drugs means an optimal approximation of the model and its superiority to corneal in vitro permeation models from other species where higher permeability factors have been described [5,6,13,15]. Therefore, HCC seems an appropriate model in in vitro corneal permeability tests predicting drug absorption into the human eye.

In order to improve corneal permeation rates and to increase the ophthalmic availability of drugs, the latter are often used as ester prodrugs. So further studies could aim at the estimation of esterase activities of HCC in comparison with the original. Furthermore toxicity studies including HCC and excised cornea will show to what extent HCC could be an alternative to the Draize eye irritancy test.

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