Ocular Pharmacokinetic Modeling Using Corneal Absorption and Desorption Rates from *in Vitro* Permeation Experiments with Cultured Corneal Epithelial Cells

Veli-Pekka Ranta,[1,2] Mirka Laavola,[1] Elisa Toropainen,[1] Kati-Sisko Vellonen,[1] Anu Talvitie,[1] and Arto Urtti[1]

Received April 10, 2003; accepted May 27, 2003

Purpose. To determine corneal absorption and desorption rate constants in a corneal epithelial cell culture model and to apply them to predict ocular pharmacokinetics after topical ocular drug application.
Method. *In vitro* permeation experiments were performed with a mixture of six β-blockers using an immortalized human corneal epithelial cell culture model. Disappearance of the compounds from the apical donor solution and their appearance in the basolateral receiver solution were determined and used to calculate the corneal absorption and desorption rate constants. An ocular pharmacokinetic simulation model was constructed for timolol with the Stella® program using the absorption and desorption rate constants and previously published *in vivo* pharmacokinetic parameters.
Results. The corneal absorption rates of β-blockers increased significantly with the lipophilicity of the compounds. The pharmacokinetic simulation model gave a realistic mean residence time for timolol in the cornea (57 min) and the aqueous humor (90 min). The simulated timolol concentration in the aqueous humor was about 1.8 times higher than the previously published experimental values.
Conclusions. The simulation model gave a reasonable estimate of the aqueous humor concentration profile of timolol. This was the first attempt to combine cell culture methods and pharmacokinetic modeling for prediction of ocular pharmacokinetics. The wider applicability of this approach remains to be seen.

KEY WORDS: ocular absorption; pharmacokinetic modeling; corneal epithelium; cell culture; cassette dosing; timolol.

INTRODUCTION

After topical ocular application, drugs may be absorbed into the inner eye through the cornea or the conjunctiva and sclera. The cornea is the main route of absorption for small, lipophilic drugs (1,2). The corneal epithelium is usually the main barrier for corneal drug permeation (3,4). Most clinically used ocular drugs have adequate lipophilicity for permeation across the cell membranes of the corneal epithelium (4,5).

The permeation of ocular drug candidates has traditionally been screened using *in vitro* experiments with excised rabbit corneas, and the compounds are ranked based on their apparent permeability coefficients (6,7). However, this parameter does not separate the two processes involved: the absorption of the compound into the cornea and the desorption from cornea into the receiver solution (or aqueous humor). Therefore, no information is obtained on the function of the corneal epithelium as a drug reservoir, even though this function is important for several drugs, e.g., pilocarpine (8) and timolol (9).

Corneal absorption and desorption rates are essential for physiologically based ocular pharmacokinetic modeling. Several pharmacokinetic models have been developed earlier using corneal absorption rates from *in vitro* permeation experiments with excised rabbit corneas (10,11). These models give useful information on the ocular pharmacokinetics of drugs, but the disadvantage concerning large-scale screening is that animals need to be sacrificed for the permeation studies with isolated rabbit corneas.

Cell culture models open the possibily of decreasing the number of animal experiments in drug transport studies (12). Recently, several corneal epithelial cell culture models have been developed based on either primary rabbit cells (13) or immortalized cell lines (14,15). Immortalized cell lines are more practical because they can be grown continuously, and the cells can be frozen and revived.

The present study decribes the first attempt to combine cell culture studies with pharmacokinetic modeling in order to predict ocular pharmacokinetics. The corneal absorption and desorption rates were determined for several β-blockers using a recently developed corneal epithelial cell culture model that is based on immortalized human cells (15). The absorption and desorption rates and several previously published pharmacokinetic parameters were applied to a simulation model in order to predict the ocular pharmacokinetics after topical application in rabbits. The model was tested with timolol, and the simulated aqueous humor concentration was fairly close to the values in the previously published *in vivo* studies.

MATERIALS AND METHODS

Cell Culture

Immortalization of human corneal epithelial (HCE) cells (16) and culturing conditions (15) have been described in detail earlier. Briefly, polyester cell culture filters (surface area 4.7 cm^2, pore size 3.0 μm; Transwell Clear, Costar, Cambridge, MA) were coated with 275 μl rat tail collagen type I (1.3 mg/ml; Becton Dickinson, Bedford, MA). Mycoplasma-free HCE cells, passages 18 to 41, in suspension were seeded onto the coated filters at a concentration of 90,000 cells/cm^2. The cells were grown at 37°C in humidified air with 5% CO_2, in standard culture medium both in apical and basolateral chambers for 7 to 10 days until the cells were confluent. The cells were then exposed to an air–liquid interface for 2 to 3 weeks. The culture medium was replaced every other day. The standard medium consisted of Dulbecco's modified eagle medium/Nutrient mix F12 (Gibco BRL, Grand Island, NY), 15% heat-inactivated fetal bovine serum (Gibco BRL), 0.3 mg/ml L-glutamine (Gibco BRL), 5 μl/ml insulin (Sigma, St. Louis, MO), 0.1 mg/ml cholera toxin (Calbiochem, La Jolla, CA), 10 ng/ml epidermal growth factor (Calbiochem), 0.5% dimethyl sulfoxide (Sigma), 0.1 mg/ml streptomycin, and 1000 IU/ml penicillin (both from Gibco BRL).

Epithelial tightness was monitored at different phases of

[1] Department of Pharmaceutics, University of Kuopio, P.O. Box 1627, FIN-70211 Kuopio, Finland.
[2] To whom correspondence should be addressed. (e-mail: velipekka.ranta@uku.fi)

0724-8741/03/0900-1409/0 © 2003 Plenum Publishing Corporation

cell growth by measuring transepithelial electrical resistance (TEER) (Endohm, World Precision Instruments, Sarasota, FL). Permeability experiments were performed with cell cultures exhibiting TEER values between 400 and 1000 $\Omega \cdot cm^2$. The cultured epithelium resembled rabbit corneal epithelium in that it consisted of five to eight cell layers and the most apical cells were flat with tight junctions, microvilli, and desmosomes (15).

Permeation Studies

β-Blockers were obtained as salts or free bases from Sigma, except that timolol maleate was kindly donated by Merck, Sharp & Dohme Research Laboratory (Rahway, NJ), and betaxolol hydrochloride by Alcon (Fort Worth, TX). A cassette dosing mixture of six β-blockers (atenolol, nadolol, pindolol, timolol, metoprolol, betaxolol) were used in the determination of absorption rates, and a mixture of eight β-blockers (additionally including sotalol and propranolol) was used in the determination of desorption rates. The mixtures were prepared in BSS Plus (balanced salt solution) (Alcon) containing 10 mM of N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) (pH adjusted to 7.4). The final concentration of each compound was 100 μM with the exception of timolol and metoprolol, which were 200 μM.

The permeation study was initiated by adding 2.6 ml of plain BSS buffer solution with 10 mM HEPES (pH 7.4) to the basolateral receiver compartment and 1.5 ml of the cassette dosing mixture to the apical donor compartment. The experiments were performed at 37°C using a horizontal plate mixer at 150 rpm (Titramax 1000 and Incubator 1000, Heidolph-Instruments, Schwabach, Germany).

In the determination of the absorption rates, aliquots of 30 μl were withdrawn from the apical compartment at 5, 10, 25, 40, and 55 min, and the samples were diluted with 270 μl of blank medium. In the determination of the desorption rates, aliquots of 200 μl were taken from the basolateral compartment at 10, 20, 30, 45, 60, 75, 90, 105, 120, 150, and 180 min and replaced with an equal volume of blank medium. Control experiments were performed with collagen-coated polyester cell culture filters without any cells. The samples were stored frozen (–20°C) until analyzed by HPLC without any extraction procedure.

During the permeation studies, the cell culture plates were kept in the incubator hood at 37°C using circulating warm air. Because the lid of the cell culture plate was removed during sampling, evaporation of the donor solution was possible. For the determination of the evaporation rate, the polyester cell culture filters were coated with a water-insoluble correction fluid (Stanger Classic, Stanger Lackchemie, Germany). The plate with the coated filters was placed into the incubator hood, and 1.5 ml of prewarmed BSS Plus solution was added to the donor chamber (receiver chamber was left empty). The lid was opened at the same time points as during the determination of the absorption rates, and the total weight of the filter and the donor solution was determined at each time point. The weight of the donor solution decreased 108 ± 3 mg (n = 3) (ca. 7.2%) between 5 and 55 min, and the change was linear (r = 0.999). The evaporation of donor solution was taken into account in the calculation of absorption rate constants.

Drug Determination

β-Blockers were analyzed simultaneously by gradient HPLC with combined ultraviolet and fluorescent detection as described earlier (17). The calibration range of the compounds ranged from 50 or 200 nM to 30 μM, and the between-day RSD of peak area within the calibration range was 2-10%.

Calculation of Kinetic Parameters

The corneal absorption rate constant (k_A, min^{-1}) was estimated from the slope of the plot of the amount of drug remaining in the donor solution vs. time. The volume of donor solution at each time point was obtained by subtracting the previous sample volumes and evaporated volume from the initial volume (1.5 ml). The clearance from the donor solution to the cornea ($CL_{D,CO}$) was obtained as

$$CL_{D,CO} = k_A V_D$$

where V_D is the average volume of the donor solution in the middle of the absorption experiment (1400 μl at 25 min). The clearance from the tear fluid via corneal absorption ($CL_{TF,CO}$) was calculated as

$$CL_{TF,CO} = CL_{D,CO}/(S_{CC}/S_{CO})$$

where S_{CC} is the surface area of the cell culture model (4.7 cm^2), and S_{CO} is the surface area of rabbit cornea (1.6 cm^2) (18).

The corneal desorption rate constant (k_D, min^{-1}) was determined using a simulation model, and this is described in the next section.

As a reference, the apparent permeability coefficient (P_{app}, cm/s) was calculated as

$$P_{app} = flux/(S_{CC} C_0)$$

where P_{app} is the apparent permeability of the epithelial cells and the collagen-coated polyester filter together, flux (nmole/s) is the linear appearance rate of the compound in the receiver solution (typically between 45 and 120 min), and C_0 (μM) is the initial concentration of the compound in the donor solution. The decline of drug concentration in the donor solution and accumulation of the drug into the cells were not taken into account in the calculation of P_{app}. This is a routine method for calculation of P_{app} in cultured epithelia (e.g., Caco-2 cells). P_{app} values were not used in the simulations.

Pharmacokinetic Simulation

Simulation models were constructed using Stella® II software (High Performance Systems, Hanover, NH) run on a Macintosh Performa 6400/200 computer. The simulations were conducted using a fourth-order Runge-Kutta algorithm with a time interval (*dt*) of 0.1 min.

The corneal desorption rate constant (k_D, min^{-1}) in the *in vitro* permeation experiment was determined using the simulation model in Fig. 1. Drug transfer from the donor solution to the cornea (nmol min^{-1}) was calculated as $k_A \times A_D$, where the absorption rate constant, k_A, was experimentally determined and fixed, and A_D is the amount of drug in the donor solution. The initial A_D was 150 or 300 nmol (1.5 ml of 100 or 200 μM solution). Drug transfer from the cornea to the receiver solution was calculated as $k_D \times A_{CO}$, where A_{CO}

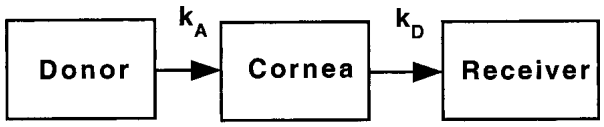

Fig. 1. The simulation model for the determination of the corneal desorption rate constant (k_D) of β-blockers in *in vitro* permeation experiments. The corneal absorption rate constant (k_A) was experimentally determined and fixed in the model. Simulations were done at various values of k_D until the best match between the observed and simulated drug concentrations in the receiver solution was found.

is the simulated amount of drug in the cornea. Correct value of k_D was obtained by simulations using the model in Fig. 1. The value of k_D was varied until the best fit between the observed and simulated drug concentrations in the receiver solution was found. In the determination of k_D, the main emphasis was given to the time period between 45 and 75 min. At these times drug permeation into the receiver solution (Fig. 2) shows a linear increase (steady state) indicating that the system is far from the thermodynamic equilibrium. For this reason, the simulation model in Fig. 1 is presented as an open system.

The simulation model for the prediction of the ocular pharmacokinetics of the drug after topical application in rabbits is shown in Fig. 3. The volume of tear fluid (V_{TF}) in the model decreases with time because of the drainage of instilled solution (19):

$$V_{TF} = V_{RES} + V_{INS} \exp(-k_{drain}\, t)$$

where V_{RES} is the resident volume of tear fluid (7.5 μl) (19), V_{INS} is the instilled volume of eye drops, and k_{drain} is the rate contant for drainage of instilled solution.

Drug transfer (μg min^{-1}) from the tear fluid is the product of the concentration of the drug in the tear fluid (C_{TF}) and

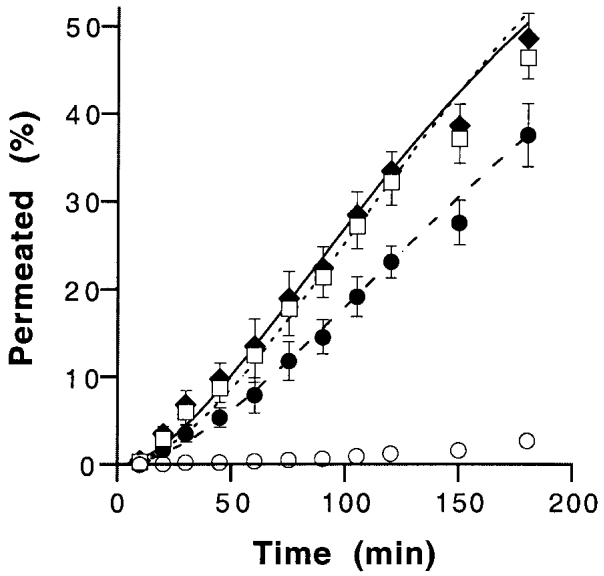

Fig. 2. Appearance of β-blockers in the receiver solution during permeation experiment with cultured corneal epithelial cells. Experimental results: (○) atenolol, (●) timolol, (♦) metoprolol, (□) betaxolol. Each point represents the mean ± SD of four culture wells in a representative experiment. Simulation: (- -) timolol (k_D = 0.0186), (__) metoprolol (k_D = 0.0260), (….) betaxolol (k_D = 0.0129). k_A values in Table I.

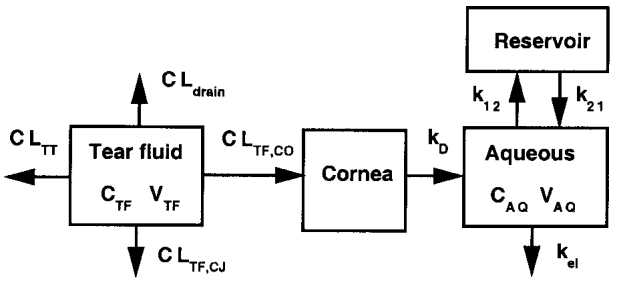

Fig. 3. The simulation model for the prediction of ocular kinetics of the drug after topical application. Abbreviation: C_{TF}, the drug concentration in the aqueous humor; V_{TF}, the volume of tear fluid; $CL_{TF,CO}$, the clearance from the tear fluid by corneal absorption; $CL_{TF,CJ}$, the clearance from the tear fluid by conjunctival absorption; CL_{TT}, the tear turnover; CL_{drain}, the clearance via drainage of instilled solution; C_{AQ}, the drug concentration in the aqueous humor; V_{AQ}, the apparent distribution volume in the aqueous humor; k_D, the corneal desorption rate constant; k_{12}, the transfer rate constant from the aqueous humor to the reservoir; k_{21}, the transfer rate constant from the reservoir to the aqueous humor; k_{el}, the elimination rate constant in the aqueous humor.

the associated clearance. Corneal absorption of the drug is determined by $CL_{TF,CO}$, and tear turnover (CL_{TT}) and conjunctival absorption ($CL_{TF,CJ}$) are nonproductive losses. The clearance from drainage of instilled solution (CL_{drain}) is another nonproductive loss factor, and its value is:

$$CL_{drain} = k_{drain}\, [V_{INS} \exp(-k_{drain}\, t)]$$

CL_{drain} declines at the same rate as the instilled volume drains from the ocular surface (the volume remaining from the instilled solution is described within the brackets in the right side of the equation). Drainage of the solution volume does not change the drug concentration in the tear fluid but causes a rapid decline in the amount of drug in the preocular area. Drug transfer from the cornea and the aqueous humor was the product of the amount of drug in the donor compartment and the associated rate constant (Fig. 3).

Timolol was used as the model compound in the simulation because the contribution of corneal route to the aqueous humor concentration is over 95% after topical application of eye drops in the rabbit (1). The simulation parameters are shown in Table II. In the simulation, the mean residence time (MRT) of timolol in the cornea and the aqueous humor was calculated as

$$MRT = AUMC/AUC$$

where AUMC is the area under the first moment curve and AUC is the area under the curve using the simulated amount of timolol in the cornea and the simulated aqueous humor concentration from 0 to infinity (MRT) or from 0 to 240 min ($MRT_{0\text{-}240}$).

RESULTS

In Vitro Permeability Experiment

The disappearance of β-blockers from the donor solution during the permeation experiment is shown in Fig. 4. The amount of hydrophilic atenolol in the donor solution decreased only slightly (by 2%) during the experiment, whereas the more lipophilic compounds were absorbed significantly

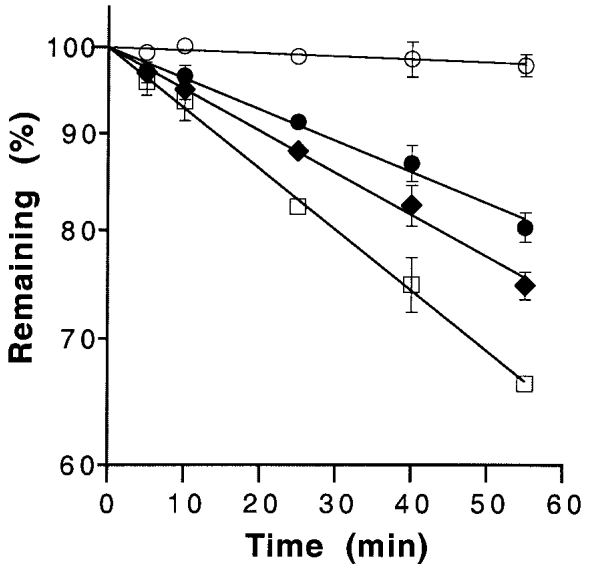

Fig. 4. Disappearance of β-blockers from the apical donor solution during the permeation experiment with cultured corneal epithelial cells. (○) atenolol, (●) timolol, (♦) metoprolol, (□) betaxolol. Each point represents the mean ± SD of 4 experiments.

into the corneal epithelial cells. The most lipophilic compound, betaxolol, was absorbed most rapidly. In the control experiments without the corneal epithelial cells, none of the compounds was significantly adsorbed on the plastic diffusion chambers (data not shown). The corneal absorption rate constants (k_A) of β-blockers are shown in Table I. The absorption rate constant could not be determined for atenolol and nadolol because of the lack of absorption. The absorption rate of pindolol was close to that of timolol.

The appearance of β-blockers in the receiver solution is shown in Fig. 2. Atenolol permeated through the epithelial cell culture very slowly suggesting that the paracellular route was dominant. Metoprolol permeated at a faster rate than betaxolol even though its absorption rate into the cells was slower.

The corneal desorption rate constants (k_D) were determined using the simulation model in Fig. 1, and the values are shown in Table I. The simulation model described the appearance rate of the drugs in the receiver solution fairly accurately up to 120 min (Fig. 2). Both the observed and simulated appearance rate curves show a lag time followed by a linear appearance rate. This is typical for permeability studies.

The simulation model in Fig. 1 also gave estimates for the amount of compounds in the corneal epithelial cells (Fig. 5). The epithelial cells contain a large amount of betaxolol that is the result of a high absorption rate and a slow desorption rate. The accumulation of betaxolol in the cells may be explained by its high distribution coefficient (Table I). The simulated amounts of timolol and metoprolol are close to each other, as are their distribution coefficients.

Table I also shows the apparent permeability coefficients (P_{app}) of the compounds. P_{app} does not give any information on the amount of compound in the epithelial cells and can not reveal the marked differences in the absorption and desorption rates between metoprolol and betaxolol.

Ocular Pharmacokinetic Modeling

The ocular pharmacokinetic simulation model in Fig. 3 with the experimental $CL_{TF,CO}$ (derived from k_A) and k_D parameters was used predict the kinetics of topically applied timolol in rabbits. Timolol was chosen as the model compound because it is absorbed mainly through the corneal route (1), and all the necessary parameters were available (Table II).

In the simulation with 25 μl of 0.5% timolol solution (dose 125 μg), timolol was absorbed from the tear fluid to the cornea during the first 5 min after instillation. During this period, the concentration of timolol in the tear fluid decreased from 3.85 μg/μl [dose /(V_{RES} + V_{INS})] to 0.04 μg/μl as a result of corneal absorption and nonproductive losses.

The simulated aqueous humor concentration was compared with the results from two *in vivo* studies where the pH of the timolol solution was similar to this study (pH 7.4–7.5) (Fig. 6). In the simulation, the maximum aqueous humor concentration of timolol was obtained at 30 min, which is within the reported range (20–30 min) in several *in vivo* studies (23–25). The maximum concentration in the simulation was slightly higher than in *in vivo* studies (Fig. 6). The simulated amount of timolol in the cornea was also higher than that observed in *in vivo* studies, e.g., 6.6 μg vs. ~3 μg (23) at 30 min after instillation.

The simulated MRT and $MRT_{0\text{-}240}$ of timolol in the cornea were 57 min and 54 min, respectively, which are close to the observed $MRT_{0\text{-}240}$ values in the cornea, 56 min (25) and

Table I. Kinetic Parameters of β-Blockers from Corneal Epithelial Cell Culture Studies

Compound	logD[a]	k_A (×10^3 min^{-1}) (n = 4)	$CL_{TF,CO}$ (μl min^{-1}) (n = 4)	k_D (×10^2 min^{-1}) (n = 4)[b]	P_{app} (×10^6 cm s^{-1}) (n = 16)
Atenolol	−1.77	–[c]	–[c]	–[c]	1.21 ± 0.39
Nadolol	−1.06	–[c]	–[c]	–[c]	1.07 ± 0.37
Pindolol	−0.07	3.66 ± 0.39	1.77 ± 0.19	1.55 ± 0.16	11.93 ± 0.66
Timolol	0.09	3.80 ± 0.48	1.83 ± 0.23	1.79 ± 0.18	12.95 ± 0.93
Metoprolol	0.03	5.07 ± 0.44	2.45 ± 0.21	2.41 ± 0.28	17.18 ± 1.28
Betaxolol	1.59	7.47 ± 0.41	3.61 ± 0.22	1.29; 1.10[d]	15.05 ± 1.68[e]

[a] Logarithm of the apparent distribution coefficient between octanol and buffer solution (pH 7.4); calculated from the data in refs. 20 and 21.
[b] Number of independent experiments each with three to six cell culture wells.
[c] Not determined becaused there was no significant absorption into the corneal epithelial cells.
[d] Values from two experiments each with four to six wells.
[e] n = 10.

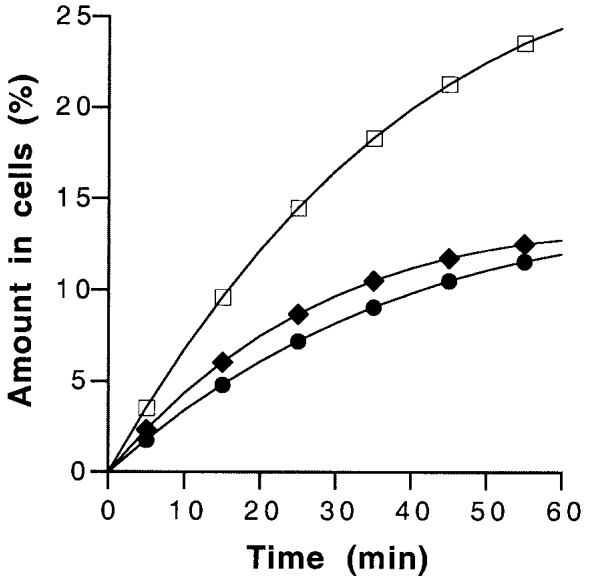

Fig. 5. Simulated amount of β-blockers in the corneal epithelial cells during the *in vitro* permeation experiment. (●) timolol, (♦) metoprolol, (□) betaxolol. The data are from a representative experiment.

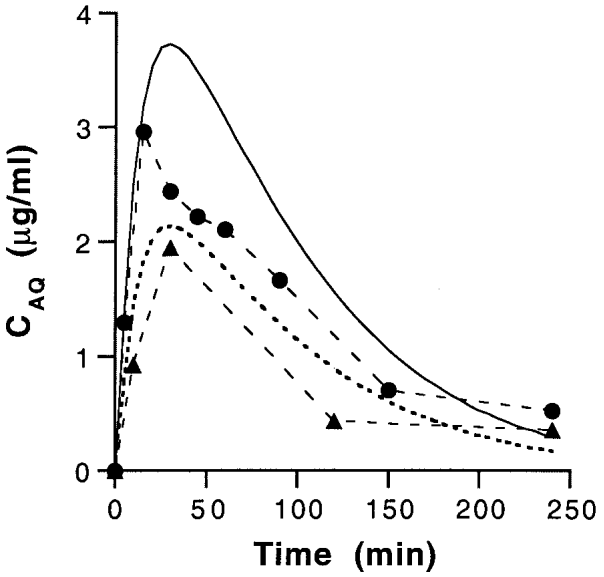

Fig. 6. Concentration of timolol in the aqueous humor after topical application of 25 μl of 0.5% solution in rabbits. Simulation: (—) using $CL_{TF,CO}$ of 1.83 μl min^{-1}, (...) using $CL_{TF,CO}$ of 1 μl min^{-1}. Other parameters in Table II. Experimental results: (▲) data from ref. 23; (●) data from ref. 9.

53 min (calculated from the data in 23), and shorter than the MRT value reported for the corneal epithelium (88 min) (9). The simulated MRT and $MRT_{0\text{-}240}$ in the aqueous humor were 90 min and 80 min, respectively, which are in the middle of the reported values in *in vivo* studies, 47 min (25), 75 min (calculated from the data in ref. 23), and 178 min (9).

The realistic MRT values in the cornea and aqueous humor suggested that deviation in the aqueous humor concentration between the simulation and the *in vivo* studies was mainly caused by the difference in the corneal absorption rate. The simulated aqueous humor concentration could be adjusted to a similar level to that in *in vivo* studies by reducing $CL_{TF,CO}$ from 1.83 to 1 μl min^{-1} (Fig. 6).

The drop size and formulation of eye drops affect the ocular bioavailability of timolol by changing the drainage rate constant and thereby the ocular contact time (Table III). The reduction of drop size from 25 μl to 5 μl reduces the dose to 20% of the initial value, but the lower drainage rate constant of 5-μl drops improves the relative bioavailability by increasing the contact time. The simulated AUC of the aqueous humor concentration curve decreased only to 30%, and an even lower reduction in bioavailability (to 45%) was observed in an *in vivo* study (Table III). The drop size of 50 μl does not improve the bioavailability by a factor of 2 because of a higher drainage rate constant. The improvement of simulated bioavailability was slightly lower than observed in *in vivo* study.

The viscous vehicle reduces the drainage rate constant, and nasolacrimal occlusion blocks the drainage completely for 10 min (Table III). These changes improved the simulated bioavailability, but the effect was not as large as observed in *in vivo* studies (Table III).

In the simulations described in Table III, the value of $CL_{TF,CO}$ was 1.83 μl min^{-1}, which causes slightly too high aqueous humor concentrations (Fig. 6). Therefore, the effect of $CL_{TF,CO}$ on the simulations was determined. When $CL_{TF,CO}$ was reduced by a factor of 3 (from 1.83 to 0.6 μl min^{-1}), the simulated relative bioavailability of the reduced drop size (5 μl) vs. the initial 25-μl drop (value of 1 in both cases) improved from 0.30 (Table III) to 0.32, and that of the nasolacrimal occlusion from 1.67 (Table III) to 1.72. These changes are modest, and they indicate that the difference in the bioavailability between the simulations and *in vivo* studies was mainly related to other kinetic parameters than $CL_{TF,CO}$. One reason for these differences is that the drainage rate constants and *in vivo* bioavailability values had to be collected from different sources. Despite these differences, the predictions of the simulation model are reasonable in most cases.

Table II. Parameters for Timolol in Ocular Pharmacokinetic Modeling

Parameter	Value	Reference
V_{TF}	$V_{RES} + V_{INS} \exp(k_{drain}\, t)$	
V_{RES}	7.5 μl	19
V_{INS}	25 μl	
k_{drain}	0.545 min^{-1}	19
CL_{drain}	$k_{drain}\, [V_{INS} \exp(k_{drain}\, t)]$	
CL_{TT}	0.53 μl min^{-1}	19
$CL_{TF,CJ}$	10.4 μl min^{-1}	22
$CL_{TF,CO}$	1.83 μl min^{-1}	This study
k_D	0.0179 min^{-1}	This study
k_{12}	0.028 min^{-1}	11
k_{21}	0.031 min^{-1}	11
k_{el}	0.057 min^{-1}	11
V_{AQ}	0.446 ml	11

DISCUSSION

Topically applied drugs may be absorbed into the eye through the corneal or conjunctival-scleral route. The cornea is the main route of absorption for small, lipophilic compounds, whereas the route through conjunctiva and sclera is important mostly for hydrophilic or large molecules (1,2). Be-

Table III. Effect of Drop Size and Formulation on the Ocular Bioavailability of 0.5% Timolol Solution[a]

Description	V_{INS} (μl)	k_{drain} (min^{-1})	Relative AUC of the aqueous humor concentration curve		Reference to *in vivo* study
			Simulation	*In vivo*	
Initial conditions	25	0.545[b]	1	1	
Reduced drop size	5	0.308[b]	0.30	0.45	27
Increased drop size	50	0.815[b]	1.33	1.67	27
Viscous vehicle[c]	25	0.1[d]	1.39	2.29	27
Nasolacrimal occlusion for 10 min	25	0 for 10 min, then 0.545[e]	1.67	2.4	28

[a] Other parameters in Table II.
[b] From ref 19.
[c] Aqueous solution containing 5% of polyvinyl alcohol.
[d] From ref 26.
[e] In addition, $CL_{TT} = 0$ because of the light anesthesia used in *in vivo* study.

cause corneal permeation is often sought in the development of new ocular drugs, it is important to develop methods for the prediction of corneal absorption.

Traditionally, ocular drug candidates have been tested using excised rabbit corneas (6,7). In the present study, corneal absorption and desorption rates were determined for several β-blockers using a recently developed corneal epithelial cell culture model that is based on immortalized human cells (15). The morphology of the cell culture model resembles rabbit corneal epithelium, and the permeability of the cell culture model for several hydrophilic and lipophilic markers is similar to that of rabbit cornea (15).

The present study describes the first attempt to combine cell culture experiments with pharmacokinetic simulation to predict ocular pharmacokinetics. Corneal absorption rate constant (k_A) was determined from the disappearance rate of the drug in the donor solution. Corneal desorption rate constant (k_D) was estimated with the simulation model (Fig. 1) in which the epithelial cells were treated as a barrier and reservoir. The simulation model was able to describe the appearance rate of the drugs in the receiver solution accurately, and both the simulated and observed curves showed a lag time followed by a linear appearance rate. The principle of using separate rate constants for absorption and desorption differs significantly from the simple calculation of the apparent permeability coefficient (P_{app}), where the cornea is treated only as a barrier but not as a depot. After topical ocular application of a drug *in vivo*, the corneal epithelium acts as a depot (8,9). Therefore, it is essential to include separate absorption and desorption rates in the model.

The experimental corneal absorption and desorption rates were integrated into a kinetic model in order to simulate the ocular kinetics of the drug after topical application in rabbits. Timolol was chosen as the model compound for the pharmacokinetic simulation because it is absorbed into the aqueous humor and iris-ciliary body predominantly through the cornea (1), and all the necessary pharmacokinetic parameters were either determined or reported in the literature.

The simulation model was fairly accurate. The simulated aqueous humor concentration of timolol was about 1.8 times higher than observed in *in vivo* studies with rabbits. The simulated MRT of timolol in the cornea and the aqueous humor were within the reported range in *in vivo* studies. In addition, the predictions on the ocular bioavailability of timolol caused by changes in drop size and formulation were reasonable in most cases.

Because the simulated aqueous humor concentration and the simulated amount of timolol in the cornea were slightly higher than those reported in *in vivo* studies, it is evident that the corneal absorption in the simulation model was higher than *in vivo*. A possible explanation might be that the experimental $CL_{TF,CO}$ from the cell culture studies is too high. The paracellular space of the cell culture is slightly higher than that in rabbit cornea, and the cell culture contains a collagen-coated polyester filter instead of corneal stroma and endothelium (15). The role of collagen-coated filter resembled that of the corneal stroma in that its permeability ($23 \pm 1 \times 10^{-6}$ cm/s, $n = 4$ for each β-blocker) was close to the values in corneal stroma of rabbits ($34 \pm 3 \times 10^{-6}$ cm/s independently of the lipophilicity of the compound) (3). These differences in paracellular route and basolateral support may affect the corneal absorption, but they are not the major sources of error in the corneal absorption rate because timolol is fairly lipophilic, and the permeability coefficient of timolol in the cell culture model ($12.95 \pm 0.93 \times 10^{-6}$ cm/s, $n = 16$) is similar to that in rabbit cornea (12.3×10^{-6} cm/s) (20,21).

The corneal absorption rate may also be affected by other factors. In the simulation with $CL_{TF,CO}$ of 1.83 μl min^{-1} (Fig. 6), it is assumed that the instilled drop is instantly mixed and spread in the lacrimal fluid over the whole corneal surface, maintaining uniform concentration on the surface. In fact, drug concentration in nonblinking rabbit eye surface may not be uniform because of solution flow to the lower cul-de-sac. Therefore, the effective surface area for drug absorption may be less than the entire corneal area. The simulation with $CL_{TF,CO}$ of 1 μl min^{-1} (Fig. 6) is obtained by reducing the effective surface area from the entire corneal area of 1.6 cm^2 to 0.9 cm^2. In this respect, the simulation with $CL_{TF,CO}$ of 1.83 μl min^{-1} describes an ideal situation and may slightly overestimate the corneal absorption.

Another explanation for the high corneal absorption rate, at least in theory, might be that the simulation model does not take into account the buildup of timolol concentration in the corneal and conjunctival epithelial surface that retards the absorption and may even lead to back-diffusion of timolol from the cornea and conjunctiva to the tear fluid at later times (29). In the simulation, the initial rate constant for the decline of timolol concentration in the tear fluid was 0.393

min^{-1} [$(CL_{TF,CO} + CL_{TF,CJ} + CL_{TT})/V_{TF}$; 12.76 μl min^{-1}/32.5 μl], and the rate constant obtained from the simulated concentrations between 0 and 1 min was 0.485 min^{-1}. These are close to the initial rate constant from *in vivo* study with rabbits (0.451 min^{-1}) (29). After 1 min, the simulated concentration of timolol in the tear fluid decreased at a faster rate than observed in *in vivo* study (29), and the simulated concentration at 5 min after instillation (0.04 μg/μl) was lower than measured in *in vivo* study (1.326 μg/μl) (29). The accuracy of the corneal absorption rate in the model could be improved by including the buildup and back-diffusion phenomena, but this would make the model much more complicated, and data on the drug concentrations in the cornea and conjunctiva and on the rate constants for back-diffusion would be required.

The rapid decline in the simulated tear fluid concentration partly explains why the increased contact time with viscous vehicle or nasolacrimal occlusion did not improve the simulated bioavailability of timolol as much as observed in *in vivo* studies. It is also likely that the viscous vehicle affects other parameters than the drainage rate constant; e.g., the clearance by conjunctival absorption ($CL_{TF,CJ}$) may be lower because of a smaller exposed area, and there may be interactions between the polymer and the drug.

Because of the lack of pharmacokinetic parameters, the simulation was not performed with other β-blockers. Nevertheless, even the corneal absorption and desorption rates of the β-blockers in the permeation experiments are useful information. For example, betaxolol has a high absorption rate and a low desorption rate, suggesting that the corneal concentrations of betaxolol are high after topical application. In fact, the high corneal concentrations of betaxolol are thought to be the reason for ocular discomfort in some patients (30). For this reason, an ion-exchange resin has been added to an ophthalmic formulation to decrease the free concentration of betaxolol in the tear fluid (30).

Earlier, several approaches have been used to develop an ocular pharmacokinetic model using the corneal absorption rate from *in vitro* experiments with excised rabbit corneas. However, the accuracy of the absorption rate in these studies is worse than in the present study with cell cultures. Miller *et al.* (10) determined the absorption rate of pilocarpine by incubating a rabbit cornea in a pilocarpine solution that was exposed to both the corneal epithelium and endothelium. The experimental clearance by corneal absorption was almost two times higher than the clearance used in the final model (1.06 vs. 0.545 μl/min). Yamamura *et al.* (11) characterized the ocular pharmacokinetics of β-blockers using a diffusion model. The authors determined the diffusion and partition parameters of timolol and tilisolol using both *in vitro* permeation experiments with excised rabbit corneas and *in vivo* experiments with rabbits. The diffusion parameters were similar in both experiments, whereas the partition parameters in the *in vitro* experiment were six to nine times higher than in an *in vivo* experiment.

Grass and Lee (31) have developed a simulation model to predict aqueous humor and plasma pharmacokinetics of ocularly applied timolol. Their model differs from the present model in that it does not contain cornea, but the drug transfer from the tear fluid to the aqueous humor is determined by a single rate constant.

The present approach may be used in the formulation studies. In these studies, the effects of the vehicle on drainage rate constant, drug release rate, and other parameters should be determined or estimated, and all these parameters should be included in the model. In the formulation studies, the ocular pharmacokinetics of the drug is often known to some extent, which improves the accuracy of the model.

This method might also be used in the screening of new drug candidates without previous *in vivo* experiments. For example, if the receptor affinity of a new drug candidate is known, the model may be used to predict if the aqueous humor concentration will be high enough to cause a pharmacologic effect. In order to get the prediction, several pharmacokinetic parameters in the model have to be estimated, e.g., the apparent distribution volume and elimination rate in the aqueous humor. Rough estimates of these parameters may be obtained based on the physicochemical properties of the drug, and the tables collected by Schoenwald (32) could be used for this purpose. This approach would probably give good enough prediction for screening purposes.

In conclusion, the present model with the experimental corneal absorption and desorption rates from the cell culture studies gives a fairly accurate estimate of the ocular pharmacokinetics of timolol. This is the first attempt to combine cell culture studies with modeling to predict ocular kinetics, and the aim is to reduce the number of animal studies. The wider applicability of the approach remains to be seen.

ACKNOWLEDGMENTS

The authors thank Kaoru Araki-Sasaki and Hitoshi Watanabe, Department of Ophthalmology, Osaka University Medical School, Japan, for kindly providing the corneal epithelial cells, and Pekka Suhonen for helpful advice. This study was aided by grants from the Academy of Finland, Finnish Cultural Foundation, Northern Savo Cultural Foundation, Kuopio University Foundation, and Finnish Graduate School in Pharmaceutical Research.

REFERENCES

1. I. Ahmed and T. F. Patton. Disposition of timolol and inulin in the rabbit eye following corneal versus non-corneal absorption. *Int. J. Pharm.* **38**:9–21 (1987).
2. D. S. Chien, J. J. Homsy, C. Gluchowski, and D. D. Tang-Liu. Corneal and conjunctival/scleral penetration of p-aminoclonidine, AGN 190342, and clonidine in rabbit eyes. *Curr. Eye Res.* **9**: 1051–1059 (1990).
3. H.-S. Huang, R. D. Schoenwald, and J. L. Lach. Corneal penetration behavior of β-blocking agents II: Assessment of barrier contributions. *J. Pharm. Sci.* **72**:1272–1279 (1983).
4. M. R. Prausnitz and J. S. Noonan. Permeability of cornea, sclera, and conjunctiva: A literature analysis for drug delivery to the eye. *J. Pharm. Sci.* **87**:1479–1488 (1998).
5. R. D. Schoenwald. Ocular drug delivery. Pharmacokinetic considerations. *Clin. Pharmacokinet.* **18**:255–269 (1990).
6. D. S. Chien, H. Sasaki, H. Bundgaard, A. Buur, and V. H. L. Lee. Role of enzymatic lability in the corneal and conjunctival penetration of timolol ester prodrugs in the pigmented rabbit. *Pharm. Res.* **8**:728–733 (1991).
7. P. Suhonen, T. Järvinen, P. Rytkönen, P. Peura, and A. Urtti. Improved corneal pilocarpine permeability with *O,O′*-(1,4-xylylene) bispilocarpic acid ester double prodrugs. *Pharm. Res.* **8**:1539–1542 (1991).
8. J. W. Sieg and J. R. Robinson. Mechanistic studies on transcorneal permeation of pilocarpine. *J. Pharm. Sci.* **65**:1816–1822 (1976).
9. V. H. L. Lee, A. M. Luo, S. Li, S. K. Podder, J. S.-C. Chang, S. Ohdo, and G. M. Grass. Pharmacokinetic basis for nonadditivity

of intraocular pressure lowering in timolol combinations. *Invest. Ophthalmol. Vis. Sci.* **32**:2948–2957 (1991).
10. S. C. Miller, K. J. Himmelstein, and T. F. Patton. A physiologically based pharmacokinetic model for the intraocular distribution of pilocarpine in rabbits. *J. Pharmacokinet. Biopharm.* **9**:653–677 (1981).
11. K. Yamamura, H. Sasaki, M. Nakashima, M. Ichikawa, T. Mukai, K. Nishida, and J. Nakamura. Characterization of ocular pharmacokinetics of beta-blockers using a diffusion model after instillation. *Pharm. Res.* **16**:1596–1601 (1999).
12. K. L. Audus, R. L. Bartel, I. J. Hidalgo, and R. T. Borchardt. The use of cultured epithelial and endothelial cells for drug transport and metabolism studies. *Pharm. Res.* **7**:435–451 (1990).
13. J.-E. Chang, S. K. Basu, and V. H. L. Lee. Air-interface condition promotes the formation of tight corneal epithelial cell layers for drug transport studies. *Pharm. Res.* **17**:670–676 (2000).
14. C. R. Kahn, E. Young, I. H. Lee, and J. S. Rhim. Human corneal epithelial primary cultures and cell lines with extended life span: *in vitro* model for ocular studies. *Invest. Ophthalmol. Vis. Sci.* **34**:3429–3441 (1993).
15. E. Toropainen, V.-P. Ranta, A. Talvitie, P. Suhonen, and A. Urtti. Culture model of human corneal epithelium for prediction of ocular drug absorption. *Invest. Ophthalmol. Vis. Sci.* **42**:2942–2948 (2001).
16. K. Araki-Sasaki, Y. Ohashi, T. Sasabe, K. Hayashi, H. Watanabe, Y. Tano, and H. Handa. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest. Ophthalmol. Vis. Sci.* **36**:614–621 (1995).
17. V.-P. Ranta, E. Toropainen, A. Talvitie, S. Auriola, and A. Urtti. Simultaneous determination of eight β-blockers by gradient high-performance liquid chromatography with combined ultraviolet and fluorescence detection in corneal permeability studies *in vitro*. *J. Chromatogr. B* **77**:81–87 (2002).
18. M. A. Watsky, M. M. Jablonski, and H. F. Edelhauser. Comparison of conjunctival and corneal surface areas in rabbit and human. *Curr. Eye Res.* **7**:483–486 (1988).
19. S. S. Chrai, T. F. Patton, A. Mehta, and J. R. Robinson. Lacrimal and instilled fluid dynamics in rabbit eyes. *J. Pharm. Sci.* **62**:1112–1121 (1973).
20. R. D. Schoenwald and H.-S. Huang. Corneal penetration behavior of β-blocking agents I: Physicochemical factors. *J. Pharm. Sci.* **72**:1266–1272 (1983).
21. W. Wang, H. Sasaki, D.-S. Chien, and V. H. L. Lee. Lipophilicity influence on conjunctival drug penetration in the pigmented rabbit: a comparison with corneal penetration. *Curr. Eye Res.* **6**:571–579 (1991).
22. I. Ahmed, R. D. Gokhale, M. V. Shah, and T. F. Patton. Physicochemical determinants of drug diffusion across the conjunctiva, sclera, and cornea. *J. Pharm. Sci.* **76**:583–586 (1987).
23. K. Järvinen, E. Vartiainen, and A. Urtti. Optimizing the systemic and ocular absorption of timolol from eye-drops. *STP Pharm. Sci.* **2**:105–110 (1992).
24. M. L. Francoeur, S. J. Sitek, B. Costello, and T. F. Patton. Kinetic disposition and distribution of timolol in the rabbit eye. A physiologically based ocular model. *Int. J. Pharm.* **25**:275–292 (1985).
25. S. Burgalassi, P. Chetoni, L. Panichi, E. Boldrini, and M. F. Saettone. Xyloglucan as a novel vehicle for timolol: Pharmacokinetics and pressure lowering activity in rabbits. *J. Ocul. Pharmacol. Ther.* **16**:497–509 (2000).
26. T. F. Patton and J. R. Robinson. Ocular evaluation of polyvinyl alcohol vehicle in rabbits. *J. Pharm. Sci.* **64**:1312–1316 (1975).
27. S.-C. Chang, D.-S. Chien, H. Bundgaard, and V. H. L. Lee. Relative effectiveness of prodrug and viscous solution approaches in maximizing the ratio of ocular to systemic absorption of topically applied timolol. *Exp. Eye Res.* **46**:59–69 (1988).
28. S.-C. Chang and V. H. L. Lee. Nasal and conjunctival contributions to the systemic absorption of topical timolol in the pigmented rabbit: Implications in the design of strategies to maximize the ratio of ocular to systemic absorption. *J. Ocul. Pharmacol.* **3**:159–169 (1987).
29. A. Urtti, J. D. Pipkin, G. Rork, T. Sendo, U. Finne, and A. J. Repta. Controlled drug delivery devices for experimental ocular studies with timolol 2. Ocular and systemic absorption in rabbits. *Int. J. Pharm.* **61**:241–249 (1990).
30. R. Jani, O. Gan, Y. Ali, R. Rodstrom, and S. Hancock. Ion exchange resins for ophthalmic delivery. *J. Ocul. Pharmacol.* **10**:57–67 (1994).
31. G. M. Grass and V. H. L. Lee. A model to predict aqueous humor and plasma pharmacokinetics of ocularly applied drugs. *Invest. Ophthalmol. Vis. Sci.* **34**:2251–2259 (1993).
32. R. D. Schoenwald. Ocular pharmacokinetics/pharmacodynamics. In A. K. Mitra (ed.) *Ophthalmic Drug Delivery Systems*, Marcel Dekker, New York, 1993, pp. 83–110.