Cornea-PAMPA as an Orthogonal in Vitro Physicochemical Model of Corneal Permeability

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Received: 21 January 2020, Accepted: 17 March 2020, Published online: 25 May 2020

Abstract
The present study was aimed to investigate the relationships between permeability and membrane retention values (log Pe and MR) of the in vitro non-cellular permeability assay, corneal-PAMPA in comparison with experimental Caco-2 permeability data and calculated physicochemical properties (MW, clogP, clogD7.4, TPSA). For the investigation, 50 structurally and physicochemically diverse drugs were selected and measured in PAMPA model optimized for corneal permeability. The results showed corneal-PAMPA model's orthogonality in terms of passive diffusion to the FDA approved Caco-2 as a gastrointestinal absorption model, while the comparison with physicochemical properties revealed trends between logPe, MR and the lipophilicity descriptors and TPSA.

Keywords
corneal-PAMPA, permeability, membrane retention, physicochemical descriptors, clogP/D7.4, TPSA

1 Introduction
The effective treatment of diseases affecting the human eye is of utmost importance as they might cause partial visual impairment or even complete blindness [1]. For the treatment of the anterior segment of the eye, topical drug administration is the preferred way of therapy. To this avail, eye drops, ophthalmic solutions, eye ointments and gels, microemulsions, drug-eluting contact lenses, ocular inserts, liposomes, nano- and microparticles etc. can be used [2–4]. However, ocular bioavailability of these topically administered drugs is limited by several factors: lacrimal fluid rapidly elutes a large portion of the applied drugs within a few minutes upon administration [5]; the drug fraction absorbed through non-corneal routes is mostly transferred into the systematic circulation [6]. These result in a decreased bioavailability (usually < 5–10 %) [7, 8], while the therapeutic effect can mainly be attributed to the drug fraction absorbed via the corneal route [6]. The human cornea is a unique and complex biological barrier which consists of five distinct layers: the epithelium, Bowman's layer, the stroma, Descemet's membrane, and the endothelium [6, 9, 10]. For the absorption of the drug into the aqueous humor it has to penetrate through these layers of hydrophilic and lipophilic character, however, it has been reported that in the case of most drugs the epithelial layer is the rate-limiting barrier [11, 12] which is responsible for about 99 % of the resistance to the diffusion of APIs through the cornea [13].

To predict the corneal permeability of APIs, several methods exist: ex vivo models using the eyes or excised cornea of vertebrate animals (most often rabbit, pig or bovine eyes) [10], while to minimize the number of laboratory animals sacrificed and to limit the cost of ex vivo studies, in vitro cellular models using primary cell cultures, immortalized cell lines, or reconstructed tissue cultures of rabbit or human origin [10, 14] are used and recently an in vitro non-cellular method, corneal-PAMPA has also been developed by our group to this avail [15].

The corneal-PAMPA method has been developed based on ex vivo rabbit corneal permeability data [7, 12] using the parallel artificial membrane permeability assay (PAMPA) [16]. Based on previous experience with the PAMPA model [17, 18], we investigated the effects of the composition of the artificial lipid membrane, the DMSO cosolvent content of the donor phase as well as different
buffer solutions in the model. We found that the best correlation ($R^2 = 0.880$) with the ex vivo data could be achieved using the following optimized conditions: iso-pH conditions using phosphate buffer saline (PBS, pH 7.4) without cosolvent, phosphatidylcholine (PC = 10.7 % (w/v); without cholesterol) dissolved in a solvent mixture of hexane:dodecane:chloroform = 70:25.5:5 (v/v) as an artificial membrane and a 4 hour long incubation of the PAMPA plates at 35 °C.

In this study, we have investigated the orthogonality between corneal-PAMPA and the FDA approved industrial cellular permeability standard, Caco-2 based on experimental data. We also aimed to investigate the extent of correlation between the experimentally determined corneal-PAMPA data (permeability ($\log P_e$) and membrane retention (MR)) and basic physicochemical properties (MW and in silico predicted $\log P$, $\log D_{298}$, $\log D_{298}$, $\log D_{298}$) to determine if it is possible to predict corneal permeability values based on these basic descriptors.

2 Experimental

2.1 Materials

Analytical grade solvents such as acetonitrile (MeCN), chloroform, dodecane, hexane and formic acid were purchased from Merck KGaA (Darmstadt, Germany). Phosphate buffered saline (PBS) powder, L-α-phosphatidylcholine (PC) and the reference materials (aldosterone, amitriptyline hydrochloride, antipyrine, atenolol, atropine sulfate, bupropion hydrochloride, carbidopa, chloramphenicol, chlorpromazine hydrochloride, clonidine, desipramine hydrochloride, diazepam, diclofenac sodium, diflunisal, diltiazem hydrochloride, ephedrine, desipramine hydrochloride, diazepam, diclofenac sodium, diflunisal, diltiazem hydrochloride, ephedrine hydrochloride, etoposide, famotidine, furosemide, haloperidol, hydrocortisone, ibuprofen, imipramine hydrochloride, irbesartan, ketoprofen, labetalol hydrochloride, lidocaine, loperamide hydrochloride, meloxicam sodium, metoprolol, nadolol, naproxen, ofloxacin, phenytoin, pindolol, pirenzepine 2 hydrochloride, piroxicam, prazosin hydrochloride, prednisolone, propranolol hydrochloride, quinine, sparfloxacin, theophylline, trimethoprim, verapamil hydrochloride, warfarin) were purchased from Sigma Aldrich Co. Ltd. (Budapest, Hungary). Further reference materials (betaxolol hydrochloride, flurbiprofen) were purchased from Toronto Research Chemicals Inc. (North York, Toronto, Canada). In all experiments, distilled water was purified by the Millipore Milli-Q® 140 Gradient Water Purification System.

2.2 Corneal-PAMPA measurements

For in vitro transcorneal permeability measurement the previously reported cornea-PAMPA method was used [15]. Briefly the drugs were dissolved in PBS buffer (pH 7.4) to make solutions of 100 μM nominal concentration. Before each assay the PBS solutions were homogenized by using an Eppendorf MixMate vortex mixer for 10–12 s and by an ultrasonic bath (Bandelin Sonorex Digiplus) for 10 min. For creation of the lipid membrane phosphatidylycerol (PC, 16 mg) was dissolved in a solvent mixture (70 % (v/v) hexane, 25 % (v/v) dodecane, 5 % (v/v) chloroform) and then each well of donor plate (MultiscreenTM-IP, MAIPN4510, pore size 0.45 mm; Millipore) was coated with the lipid solution (5 μL each). Then the hexane and chloroform were evaporated to form a PC lipid membrane with the concentration of 10.67 w/v% in each well. Then the donor plate was fit into the acceptor plate (Multiscreen Acceptor Plate, MSAACCECTOR; Millipore) containing 300 μL of PBS solution (pH 7.4), and 150–150 μL of the PBS solutions were put on the membrane of the donor plate. The donor plate was covered with a sheet of wet tissue paper and a plate lid to avoid evaporation. The plates were incubated for 4 h at 35 °C (Heidolph Titramax 1000) followed by separation of PAMPA sandwich plates and determination of concentrations of the APIs in the donor and acceptor solutions by HPLC-DAD. The concentration of donor solutions at time point zero was also determined using the same HPLC system. Test solutions from PAMPA experiments were prepared in 96-well plates and sealed before injection. For each assay 3 replicates per compounds were measured.

The effective permeability and membrane retention of drugs were calculated using Eq. (1) [16]:

$$P_e = -\frac{2.303}{A} \times \left(1 + r_e \right) \times \log \left[ -r_e \times \left( 1 + r_e \right) \times \frac{C_D(t)}{C_D(0)} \right],$$

(1)

where $P_e$ is the effective permeability coefficient (cm/s), $A$ is the filter area (0.3 cm²), $t$ is the incubation time (s), $r_e$ is the time to reach steady-state (s), $r_v$ is the volume ratio of aqueous compartments ($V_o/V_v$), $V_o$ and $V_v$ are the volumes in the donor (0.15 cm³) and acceptor phase (0.3 cm³), $c_D(t)$ is the concentration of the compound in the donor phase at time point $t$ (mol/cm³), $c_D(0)$ is the concentration of the compound in the donor phase at time point zero (mol/cm³) and MR is the membrane retention factor, defined as [16]:

$$MR = 1 - \frac{C_D(t)}{C_D(0)} = \frac{V_v \times C_D(t)}{V_o \times C_D(t)},$$

(2)
where $c_A(t)$ is the concentration of the compound in the acceptor phase at time point $t$ (mol/cm$^3$).

### 2.3 HPLC-methods

Quantitative chromatographic analyses were performed using an Agilent 1260 liquid chromatography system equipped with a vacuum degasser, a quaternary pump, a thermostatted autosampler, a column temperature controller and a diode array detector (Agilent Technologies, Palo Alto, CA, USA) at 45 °C on a Kinetex® 2.6 µm C18 100 Å LC column (30 x 3 mm) with a mobile phase flow rate of 1.1 mL/min. Composition of mobile phase A was 0.1 % (v/v) formic acid in water, B was MeCN/water 95/5 (v/v) with 0.1 % (v/v) of formic acid. A 3.91 min long, linear gradient program was applied: 0 % B in the first 0.3 min, 0–100 % B between 0.3 and 1.8 min, then 100 % B was kept for another 0.6 min, and finally at 2.41 min the percentage of B was dropped to 0 %. This was followed by an equilibration period of 1.5 min prior to the next injection. Chromatograms were recorded at the wavelength of 200–500 nm, integration was carried out using ChemStation B.04.03 was used for data acquisition and analysis.

### 3 Results and discussion

For our experiments, we have selected fifty APIs of commercially available drugs (Table 1) with diverse molecular structure and physicochemical parameters covering a broad range of molecular weight (MW = 165 – 589), lipophilicity descriptors (clog$P = -2.0 – 5.4$, clog$D_{7.4} = -2.7 – 4.2$) and topological polar surface area (TPSA = 3 – 176 Å$^2$). Table 1 also contains the in vitro corneal permeability and membrane retention values measured by the corneal-PAMPA method [15], as well as previously reported experimental Caco-2 permeability data [19–21].

As we can see in Fig. 1 only a weak correlation could be observed between the in vitro experimental corneal-PAMPA and Caco-2 permeability values (Pearson correlation coefficient, $r = 0.354$), which indicates that our model is independent of this generally accepted gastrointestinal permeability model and supports its adequacy for cornea-specific in vitro measurements.

To investigate the correlation of corneal permeability values and basic physicochemical parameters clog$P$, clog$D_{7.4}$ and TPSA values have been predicted using the Marvin calculator plugin [22] (Table 1). Determination of correlation coefficients and model fitting was carried out using GraphPad Prism v.7.03. [23].

### Table 1 In silico predicted physicochemical parameters and experimental corneal-PAMPA and Caco-2 permeability values of investigated APIs

| API name          | MW     | clog$P$ | clog$D_{7.4}$ | TPSA$^*$ (Å$^2$) | $P_e$ (10$^6$ cm/s) | Corneal-PAMPA       | Caco-2       |
|-------------------|--------|---------|--------------|------------------|---------------------|---------------------|--------------|
| aldoosterone      | 360.45 | 1.06    | 1.06         | 91.67            | 9.05                | 0.12                | -5.04       | 4.06  | 1.65  | 48.98  | -4.31  | [19] |
| amitriptyline     | 277.41 | 4.81    | 2.48         | 3.24             | 24.52               | 2.43                | -6.61       | 73.36 | 1.04  | 20.89  | -4.68  | [19] |
| antipyrine         | 188.23 | 1.22    | 1.22         | 23.55            | 15.46               | 2.15                | -5.81       | 1.50  | 0.40  | 28.18  | -4.55  | [20] |
| atenolol          | 266.34 | 0.43    | -1.00        | 84.58            | 0.00                | -                  | -           | 2.58  | 0.44  | 0.36   | -6.44  | [20] |
| atropine          | 289.38 | 1.57    | -0.41        | 49.77            | 6.81                | 1.97                | -5.18       | 3.93  | 0.36  | 19.50  | -4.71  | [19] |
| betaxolol         | 307.43 | 2.54    | 0.31         | 50.72            | 23.67               | 2.82                | -4.63       | 10.31 | 2.17  | 30.20  | -4.52  | [20] |
| bupropion         | 239.74 | 3.27    | 2.39         | 29.10            | 32.65               | 0.30                | -4.47       | 34.44 | 2.38  | 151.36 | -3.82  | [21] |
| carbamazepine     | 236.27 | 2.77    | 2.77         | 46.33            | 29.68               | 3.15                | -4.53       | 8.26  | 1.91  | 5.01   | -5.30  | [20] |
| chloramphenicol   | 323.13 | 0.88    | 0.86         | 112.70           | 2.53                | 0.08                | -5.60       | 8.40  | 0.61  | 20.42  | -4.69  | [20] |
| chloropramazine   | 318.86 | 4.54    | 2.74         | 6.48             | 6.73                | 2.31                | -5.19       | 86.24 | 0.98  | 19.95  | -4.70  | [20] |
| cinmetidine       | 252.34 | -0.11   | -0.22        | 88.89            | 0.00                | -                  | -           | 1.50  | 1.29  | 1.29   | -5.89  | [20] |
| ciprofloxacin     | 331.35 | -0.86   | -0.87        | 72.88            | 0.25                | 0.19                | -6.70       | 4.21  | 1.36  | 2.95   | -5.53  | [21] |
| clonidine         | 230.09 | 2.49    | 1.66         | 36.42            | 24.05               | 2.40                | -4.62       | 6.91  | 4.97  | 25.70  | -4.59  | [20] |
| desipramine       | 266.39 | 3.90    | 1.37         | 15.27            | 54.42               | 1.33                | -4.26       | 27.46 | 3.05  | 21.38  | -4.67  | [20] |
| diazepam          | 284.74 | 3.08    | 3.08         | 32.67            | 60.28               | 2.70                | -4.22       | 38.90 | 3.86  | 47.86  | -4.32  | [20] |
| diclofenac        | 296.15 | 4.26    | 1.10         | 49.33            | 19.96               | 2.63                | -4.70       | 8.33  | 1.81  | 53.70  | -4.27  | [21] |
| diffenusal        | 250.2  | 3.91    | 0.41         | 57.53            | 5.84                | 0.36                | -5.23       | 14.29 | 0.79  | 12.59  | -4.90  | [19] |
| diltiazem         | 414.52 | 2.73    | 1.89         | 59.08            | 44.73               | 0.40                | -4.35       | 28.49 | 4.44  | 41.69  | -4.38  | [20] |
| ephedrine         | 165.24 | 1.32    | -0.78        | 32.26            | 12.18               | 1.26                | -4.92       | 8.85  | 0.51  | 10.23  | -4.99  | [19] |
| etoposide         | 588.56 | 1.16    | 1.16         | 160.83           | 0.73                | 0.09                | -6.14       | 8.38  | 1.74  | 1.00   | -6.00  | [21] |
Table 1. Correlation between permeability and molecular descriptors, only a weak correlation could be observed with MW (Fig. 2 (A)), while the comparison revealed a moderate correlation with \( \text{clog} D_{7.4} \) (Fig. 2 (E)), a strong positive correlation in the case of \( \text{clog} P \) (Fig. 2 (C)) and a strong negative correlation with TPSA (Fig. 2 (G)). These are in agreement with the fact that the more lipophilic a drug is, the easier it can partition into the membrane (PC, in our model).

On the other hand, a large polar surface area will hinder it can partition into the membrane (PC, in our model). Therefore a negative trend can be expected.

![Diagram](image_url)

**Fig. 1** Correlation between experimental corneal-PAMPA and Caco-2 permeability values, \( r \) is the Pearson correlation coefficient [23].
Fig. 2 Correlation between experimental corneal permeability ($\log P_e$), membrane retention (MR) and in silico predicted physicochemical parameters ($\log P$: the logarithm of the partition coefficient, $\log D_{7.4}$: the logarithm of the distribution coefficient at pH 7.4, TPSA: topological polar surface area). Physicochemical parameters were predicted by the Chemaxon/Marvin Sketch 19.19.0 plugin [22]. $r$ is the Pearson correlation coefficient, $R^2$ is the goodness of fit of the fitted model calculated using GraphPad Prism v. 7.03 [23].

lines fitted onto the datapoint using linear regression analysis were low, only poor fits could be achieved in all cases. Similar observations could be made in the case of MR values: it showed a very weak correlation with MW (Fig. 2 (B)), moderately strong correlation with the lipophilicity descriptors ($\log P$ and $\log D_{7.4}$) and TPSA (Fig. 2 (H)). The relationship between MR and the lipophilicity descriptors seemed to be non-linear, instead a sigmoidal model could be fitted over the datapoints, although it showed only a poor fit. In the case of TPSA and MR a fair fit could be observed using an exponential model.

The observed relationships between $\log P_e$, MR and the predicted physicochemical descriptors show that using only one basic parameter separately would not result
in precise prediction of corneal permeability. To improve the
goodness of in silico prediction, a larger dataset of mea-
sured permeability values would be needed to carry out a
QSPR analysis that uses further physicochemical descriptors.

4 Conclusions
Due to the particularly low bioavailability of ophthal-
mic formulations, the prediction of corneal permeability
is essential from the early stage of drug discovery. To this
avail a high throughput, non-cellular in vitro permeability
assay, corneal-PAMPA has been developed. In the current
study, we compared corneal-PAMPA permeability data of
fifty APIs with experimental Caco-2 permeability values,
which showed that the two models are independent of each
other. We also investigated the relationship between \( \log P \)
and MR values and four basic physicochemical descriptors,
MW, clog\( P \), clog\( D_{\text{av}} \), and TPSA. Based on the results, we can
conclude that although noticeable and obvious trends could
be observed between the experimental corneal-PAMPA val-
ues (\( \log P \), MR) and the lipophilicity and TPSA descriptors,
the correlations with separate parameters alone were not
strong enough for precise prediction of corneal permeabil-
ity. To this avail, in the future in the possession of a larger
experimental dataset of corneal-PAMPA values a thorough
QSPR analysis may be carried out.

Acknowledgement
This research was funded by the New National Excellence
Program of the Ministry of Human Capacities (grant number ÚNKP-19-2-I-BME-370).

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