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Revisiting the pK_a-Flux method for determining intrinsic membrane permeability

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ABSTRACT

Intrinsic membrane permeability is one of several factors that critically determine the intestinal absorption of a chemical. The intrinsic membrane permeability of a chemical is usually extracted from transwell experiments with Caco-2 or MDCK cells, preferably by the pK_a-Flux method, which is considered the method of choice when aqueous boundary layer effects need to be excluded. The pK_a-Flux method has two variants, the iso-pH method, where apical and basolateral pH are equal, and the gradient-pH method, where apical and basolateral pH are different. The most commonly used method is the gradient-pH method, as it is intended to reflect the pH-conditions in the gastrointestinal tract. However, concentration-shift effects caused by the applied pH-difference between apical and basolateral compartment in the gradient-pH method have not been considered in the evaluation of the experimental data in the past. Consequently, incorrect intrinsic membrane permeabilities have been determined. In this work, we present a revised method for extracting the intrinsic membrane permeability from gradient-pH data that considers concentration-shift effects in the basolateral aqueous boundary layer and filter as well as in the cytosol. Furthermore, we propose the use of the iso-pH method, where only concentration-shift effects in the cytosol need to be considered, as an alternative to the gradient-pH method. We use the five lipophilic bases amantadine, chloroquine, propranolol, venlafaxine and verapamil as examples to compare gradient-pH method and iso-pH method with regard to the extractability of the intrinsic membrane permeability. For lipophilic bases, the iso-pH method proves to be advantageous. All intrinsic membrane permeabilities determined in this work were substantially higher than the intrinsic membrane permeabilities reported in literature.

1. Introduction

Knowledge of the intestinal absorption of chemicals is key to the successful development of orally administered drugs (Youdim et al., 2003). The *in vitro* gold standard for predicting the intestinal absorption of chemicals are transwell experiments with human colorectal adenocarcinoma (Caco-2) or Madin-Darby canine kidney (MDCK) cells. In these experiments, two compartments, apical and basolateral, are separated by a confluent cell monolayer grown on a permeable filter support (Hubatsch et al., 2007).

In order to diffuse from one compartment to the other in this setup, a chemical has to pass several permeation barriers in series (see Fig. 1): the apical aqueous boundary layer (ABL,a), the cell monolayer, the filter and the basolateral aqueous boundary layer (ABL,b). The chemical can pass through the cell monolayer via two parallel pathways: the paracellular pathway (para) through water-filled pores between the cells and the transcellular pathway (trans) through the apical membrane (m,a), cytosol (cyt) and basolateral membrane (m,b) of the cells. In theory, there is a third pathway, lateral diffusion within the membrane (Bittermann and Goss, 2017; Sawada et al., 1999). We will not discuss this lateral pathway further, as it has turned out to be negligible for the vast majority of compounds, including the compounds investigated in this study (Bittermann and Goss, 2017). The individual permeabilities (P) of all permeation barriers contribute to the total apparent permeability (P_{app}), that is measured in the transwell setup (Avdeef et al., 2005; Bittermann and Goss, 2017).

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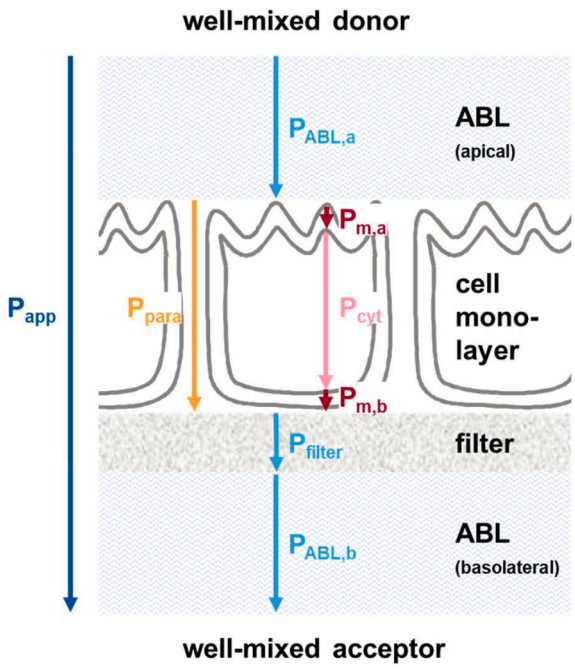

Fig. 1. Permeation barriers and associated permeabilities in Caco-2 and MDCK transwell assays. Well-mixed donor and well-mixed acceptor compartment are separated by apical ABL, cell monolayer, filter and basolateral ABL. The chemical can pass through the cell monolayer via the transcellular route (including apical membrane, cytosol and basolateral membrane) and the paracellular route. The lateral route is considered irrelevant and therefore not depicted.

$$P_{app} = \frac{1}{\frac{1}{P_{ABL,a}} + \frac{1}{P_{trans,a\rightarrow b} + P_{para}} + \frac{1}{P_{filter}} + \frac{1}{P_{ABL,b}}} \quad (1)$$

With:

$$P_{trans,a\rightarrow b} = \frac{1}{\frac{1}{P_{m,a}} + \frac{1}{P_{cyt}} + \frac{1}{P_{m,b}}} \quad (2)$$

Although transwell experiments with Caco-2 or MDCK cells attempt to mimic the *in vivo* situation, the P_{app} determined in these *in vitro* experiments cannot directly be compared to the P_{app} *in vivo* (Avdeef and Tam, 2010). *In vivo*, for example, no filter is present and the thicknesses of the ABLs, especially the basolateral ABL, are substantially reduced (Avdeef et al., 2004).

In order to be able to transfer *in vitro* data to the *in vivo* situation, it is therefore necessary to thoroughly understand and quantify each individual sub-process contributing to P_{app}. The permeation through aqueous barriers such as the ABL, the cytosol and the water-filled filter pores is assumed to be well understood (Avdeef, 2012; Bittermann and Goss, 2017). P_{para} is only relevant for very hydrophilic compounds such as sugars or ions (Artursson et al., 2012). Therefore, the main focus of research is on P_m.

P_m can only be extracted from P_{app}, if P_{app} is neither dominated by diffusion through ABL and filter nor by paracellular transport. In order to exclude the effects of ABL, filter and paracellular transport on the extracted P_m, the pK_a-Flux method was introduced by Avdeef et al. (2005). In this method, P_{app} is determined at multiple pH values. There are two variants of the pK_a-Flux method: the iso-pH method, where apical and basolateral pH are varied simultaneously, and the gradient-pH method, where only the apical pH is varied while the basolateral pH is kept constant at pH 7.4 (Avdeef, 2012). Predominantly, the gradient-pH method is used because it is supposed to represent the pH-conditions in the gastrointestinal tract (Avdeef et al., 2005; Nagahara et al., 2004; Neuhoff et al., 2003; Neuhoff et al., 2005; Palm et al., 1999). Plotting log P_{app} against apical pH usually gives a sigmoidal curve, with the upper plateau representing log P_{ABL} and the lower plateau representing log P_{para} (Lozoya-Agullo et al., 2017). The diagonal section between both plateaus is assumed to contain a substantial contribution of P_m and is therefore used for the extraction of P_m (Lozoya-Agullo et al., 2017).

This data evaluation is based on the knowledge that P_m is pH-dependent for ionizable chemicals because, according to the pH-partition hypothesis, only the neutral fraction (f_n) of a compound is able to pass through membranes. For better comparability, P_m is therefore usually divided by the neutral fraction at the respective apical pH to obtain the pH-independent intrinsic membrane permeability (P_0) of the neutral species:

$$P_0 = \frac{P_m}{f_n} \quad (3)$$

However, the use of pH-differences between apical and basolateral compartment in permeability assays is associated with difficulties. Some of these difficulties have already been considered in literature. When using the gradient-pH method, Neuhoff et al. (2003) found efflux ratios that are not caused by active transport but by the pH-difference between apical and basolateral compartment. The effects of pH-differences on sink conditions and retention have been described by Avdeef (2012). However, there is an additional problem with the evaluation of pK_a-Flux data, especially gradient-pH data, that has not yet been considered: the occurrence of pH-dependent concentration-shift effects causing the permeability of the aqueous barriers to change as a function of pH. This is in direct contrast to the previous assumption that the permeability of the aqueous barriers is independent of pH.

With regard to these concentration-shift effects in aqueous barriers, the aims of this study were to (i) experimentally demonstrate the differences between the gradient-pH method where only the apical pH is varied, and the iso-pH method where both apical and basolateral pH are varied simultaneously, (ii) point out why the established method for extracting P_0 from pK_a-Flux-data is incorrect, (iii) present mathematical equations for the correct extraction of P_0 from both the gradient-pH and iso-pH method and (iv) show the advantages of the iso-pH method over the gradient-pH method in the extraction of P_0 of lipophilic bases.

In addition, it is important to note that it is widely assumed that cells in transwell assays maintain a constant cytosolic pH (pH_{cyt}) of 7.4 independent of the external pH (pH_e) (Heikkinen et al., 2010; Neuhoff et al., 2005). However, literature data suggest that pH_{cyt} changes depending on the pH_e in MDCK (Borle and Bender, 1991; Chatton and Spring, 1994) as well as Caco-2 cells (Liang et al., 2007; Michl et al., 2019; Perdikis et al., 1998). For a correct extraction of P_0 from experimental data, knowledge of pH_{cyt} is required as we will show in the respective mathematical equations. Consequently, our last aim was to (v) determine the pH_{cyt} of MDCK cells as a function of pH_e and discuss the consequences for evaluation of the experimental data. So far this relationship between pH_{cyt} and pH_e in MDCK cells has only been investigated for pH_e between 6.8 and 8 (Borle and Bender, 1991; Chatton and Spring, 1994). Here, we extend this range because pH_e between 5 and 8 are frequently used in Caco-2 and MDCK transport experiments (Avdeef et al., 2005; Nagahara et al., 2004; Neuhoff et al., 2003; Neuhoff et al., 2005; Palm et al., 1999).

2. Theoretical section

For ionizable compounds, the occurrence of pH-differences in the transwell system is associated with the occurrence of concentration-shift effects. Eqs. (1) and (2) are therefore only applicable to a system in which the pH is the same in each aqueous barrier. If the pH in the apical ABL is different from the pH in the cytosol or the pH in the basolateral ABL and filter, additional concentration-shift factors ($S_x^{a\rightarrow b}$) must be included in the calculation of P_{app}:

$$P_{app} = \frac{1}{\frac{1}{P_{ABL,a}} + \frac{1}{P_{trans,a\to b} + P_{para}} + \frac{1}{S^{a\to b}_{filter} \cdot P_{filter}} + \frac{1}{S^{a\to b}_{ABL,b} \cdot P_{ABL,b}}} \quad (4)$$

With:

$$P_{trans,a\to b} = \frac{1}{\frac{1}{P_{m,a}} + \frac{1}{S^{a\to b}_{cyt} \cdot P_{cyt}} + \frac{1}{P_{m,b}}} = \frac{1}{\frac{1}{24 f_{n,a} \cdot P_0} + \frac{1}{S^{a\to b}_{cyt} \cdot P_{cyt}} + \frac{1}{f_{n,a} \cdot P_0}} \quad (5)$$

In addition to the concentration-shift factors, a factor of 24 is included in the calculation of $P_{m,a}$ to account for the increased surface area of the apical membrane due to microvilli (Palay and Karlin, 1959). The concentration-shift factors can be expressed as follows:

$$S^{a\to b}_{cyt} = \frac{f_{n,a}}{f_{n,cyt}} \quad (6)$$

$$S^{a\to b}_{ABL,b} = S^{a\to b}_{filter} = \frac{P_{trans,a\to b} + P_{para}}{P_{trans,b\to a} + P_{para}} \quad (7)$$

With:

$$P_{trans,b\to a} = P_{trans,a\to b} \cdot \frac{f_{n,b}}{f_{n,a}} \quad (8)$$

The mathematical derivation of the concentration-shift factors and the calculation of the neutral fractions is described in detail in Fig. S1 and Eqs. (S1)–(S14) in the Supporting Material. In the following, the pH-dependent permeabilities of cytosol, filter and basolateral ABL are referred to as P^S_{cyt}, P^S_{filter} and $P^S_{ABL,b}$ to emphasize that the concentration-shift factor is included in the calculation of the respective permeability.

A concentration-shift effect in the cytosol can occur regardless of whether the iso-pH method or the gradient-pH method is used. The concentration-shift effect is based on the fact that only the neutral species of a compound can pass through a membrane. The membrane permeability of the ionic species is usually negligible. Yet the total concentration (neutral species plus ionic species) contributes to the diffusion through aqueous layers. The mathematically derived concentration-shift effect in the cytosol is best visualized using an extreme situation: Assuming that the membrane does not present a substantial resistance to permeation, the concentration of the neutral species in the apical compartment and the cytosol adjacent to the membrane will equalize quickly. If pH_a and pH_{cyt} are different, the dissociation and consequently the total concentration of the compound is different in each compartment, which directly affects the diffusion through the cytosol. The concentration-shift factor in the cytosol can therefore be expressed as the ratio of the total concentration in the cytosol and the apical compartment which corresponds to the ratio of the fraction of the neutral species in the apical compartment ($f_{n,a}$) and the cytosol ($f_{n,cyt}$) (see Eq. (6)). If pH_a and pH_{cyt} are equal, $S^{a\to b}_{cyt}$ is equal to 1. The larger the difference between pH_a and pH_{cyt} the more pronounced the concentration-shift effect in the cytosol. In order to calculate $S^{a\to b}_{cyt}$, exact knowledge of pH_{cyt} is required.

Due to the pH-difference between apical and basolateral compartment, an additional concentration-shift effect occurs in the basolateral ABL and the filter when the gradient-pH method is used. Again, this effect is best visualized using extreme situations: (i) If a compound is exclusively transported via the paracellular route, no concentration-shift effect occurs because both the neutral and ionic species of a compound are transported paracellularly. Consequently, $S^{a\to b}_{ABL,b}$ and $S^{a\to b}_{filter}$ are equal to 1. (ii) If a compound is exclusively transported via the transcellular route and the cell monolayer does not present a substantial resistance to permeation, again, we can assume equal neutral concentrations on both sides adjacent to the cell monolayer. The concentration-shift factors $S^{a\to b}_{ABL,b}$ and $S^{a\to b}_{filter}$ could then be expressed analogous to the cytosol as the ratio of the fraction of the neutral species in the apical compartment ($f_{n,a}$) and the basolateral compartment ($f_{n,b}$). However, often both the transcellular and paracellular pathway contribute to the concentration-shift effect.

A distinction is made between positive and negative concentration-shift effects. A positive concentration-shift effect is characterized by a concentration-shift factor larger than 1, resulting in an increased concentration and consequently an increased permeability. In reverse, a negative concentration-shift effect is characterized by a concentration-shift factor less than 1, resulting in a decreased concentration and consequently a decreased permeability in the respective aqueous compartment. For this reason, especially the negative concentration-shift effect poses a problem in the extraction of P_0. Although P_0 itself is not affected by concentration-shift effects and therefore independent of the applied method and pH-conditions, the negative concentration-shift effects increase the relative contributions of cytosol, basolateral ABL and filter and therefore decrease the relative contribution of the membrane to the total permeation process, which negatively affects the extractability of P_0.

Regarding transwell experiments, it is usually assumed that pH_{cyt} remains constant at 7.4. When the gradient-pH method is used, the pH in the basolateral compartment also remains constant at 7.4. Consequently, for bases, the concentration-shift effect in the cytosol, basolateral ABL and filter is negative for pH_a less than 7.4 and positive for pH_a larger than 7.4. The opposite is true for acids. When the iso-pH method is used, the pH in the basolateral compartment is equal to the pH in the apical compartment. Consequently, there is no concentration-shift effect in the basolateral ABL and filter, and only the concentration-shift effect in the cytosol needs to be considered. The different scenarios with the assumption of a constant pH of 7.4 in the cytosol are visualized in Fig. S2A in the Supporting Material.

In order to extract P_0 from P_{app}, the contribution of each sub-process needs to be determined separately. P_{ABL} can be predicted from the diffusion coefficient of the chemical in water (D_w), which can be estimated from the molecular weight (MW) of the compound, and the thickness of the ABL (x_{ABL}) (Avdeef et al., 2005; Avdeef, 2010):

$$P_{ABL} = \frac{D_w}{x_{ABL}} \quad (9)$$

With:

$$D_w = 1.348 \cdot 10^{(-4.13 - 0.453 \cdot \log(MW))} \quad (10)$$

P_{cyt} can be predicted analogously to P_{ABL} with the diffusion coefficient of the chemical in the cytosol (D_{cyt}), which is estimated to be one quarter of D_w (Verkman, 2002), and the thickness of the cytosol (x_{cyt} = 15 μm) (Bittermann and Goss, 2017):

$$P_{cyt} = \frac{D_{cyt}}{x_{cyt}} \quad (11)$$

P_{filter} can be calculated from the pore density (n), the pore radius (r_p), the diffusion coefficient in water (D_w) and the filter thickness (x_{filter}) (Karlsson and Artursson, 1991):

$$P_{filter} = \frac{n \pi r_p^2 D_w}{x_{filter}} \quad (12)$$

P_0 and P_{para} can be derived from a fit of P_{app} according to Eq. (4).

3. Material and methods

3.1. Cell culture

MDCK-II-wildtype cells were obtained from The Netherlands Cancer Institute (Amsterdam, The Netherlands) at passage 9. The cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in an atmosphere of 5 % CO_2. The cells were passaged twice a week.

3.2. Transwell experiments

3.2.1. Selection of test compounds

Since the impact of negative concentration-shift effects on the extraction of P_0 is most pronounced with lipophilic bases, we selected five lipophilic bases based on their hexadecane-water partition coefficient ($K_{hex/w}$) (Bittermann and Goss, 2017) predicted by the UFZ-LSER database (Ulrich et al., 2017): amantadine (log $K_{hex/w}$: 1.22), chloroquine (log $K_{hex/w}$: 3.21), propranolol (log $K_{hex/w}$: 0.57), venlafaxine (log $K_{hex/w}$: 0.84) and verapamil (log $K_{hex/w}$: 1.41).

3.2.2. Transport experiments

For transport experiments, MDCK cells (passage: 23 to 33) were seeded on 12-Well PET Inserts (CellQART, Northeim, Germany; pore density: 1×10^8 pores/cm^2; pore radius: 0.2 μm; filter thickness: 11.5 μm; filter area: 1.1 cm^2; porosity: 0.13) at a density of 2.5×10^5 cells/insert. The cells were maintained as described in Section 3.1 for three days. The culture medium was renewed one day before the transport experiments to ensure optimal nutrient supply.

Prior to the transport experiments, the inserts were washed with Hanks' balanced salts solution (HBSS) to remove residual DMEM. In order to inhibit active transport, the inserts were preincubated for 30 min with HBSS pH 7.4 containing 2 μM elacridar.

The apical-to-basolateral (a→b) transport of the five test compounds was determined in duplicate under different pH conditions. Two different methods were used: 1) The iso-pH method, where the same pH is used in the apical and basolateral compartment (pH 5→5, 5.5→5.5, 6→6, 6.5→6.5, 7→7, 7.4→7.4, 8→8) and 2) The gradient-pH method, where only the apical pH is varied and a constant pH of 7.4 is used in the basolateral compartment (pH 5→7.4, 5.5→7.4, 6→7.4, 6.5→7.4, 7→7.4, 8→7.4). For pH 5 to 6.5 HBSS buffered with 10 mM MES was used. For pH 7 to 8 HBSS buffered with 25 mM HEPES was used. Stock solutions of the test compounds (5 μg/ml – 10 μg/ml) in the respective HBSS were prepared. The pH was controlled before and after the experiments with a Rapid_pH automated pH Meter (Hudson Robotics, Inc., Springfield, NJ, USA). Throughout the experiment, the pH was constant within a range of ± 0.1 for pH 5, 5.5, 6, 7, 7.4 and 8 and within a range of ± 0.2 for pH 6.5.

The transport experiments were conducted with 0.5 ml stock solution containing 2 μM elacridar in the apical donor compartment and 1.6 ml HBSS in the basolateral acceptor compartment. The well plates were placed on a temperature-controlled Titramax 1000 orbital shaker (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) set to 37 °C and 450 rpm to reduce the thickness of the ABL. Samples from the basolateral acceptor compartment were taken after 10, 20 and 30 min by transferring the inserts with the stock solution to new well plates containing 1.6 ml fresh HBSS buffer. Samples from the apical donor compartment were taken after 30 min to calculate the recovery. The samples were analyzed using an Infinity II 1260 LC system coupled to a 6420 triple quadrupole with ESI source (Agilent Technologies Inc., Santa Clara, USA). Sink conditions were maintained throughout the whole experiment.

3.2.3. Calculation of experimental p_{app}

P_{app} [cm/s] was calculated from the acceptor concentrations $c_{A,tx}$ and $c_{A,tx-1}$ [μg/ml] measured at the two consecutive time points t_x and t_{x-1} [s]:

$$\boldsymbol{P_{app}} = \frac{\boldsymbol{c_{A,tx} - c_{A,tx-1}}}{\boldsymbol{t_x - t_{x-1}}} \cdot \frac{\boldsymbol{V_A}}{\boldsymbol{A \cdot \Delta c}} \tag{13}$$

Where V_A [cm^3] is the volume of the basolateral acceptor compartment, A [cm^2] is the filter area and Δc [μg/ml] is the concentration difference between apical donor and basolateral acceptor compartment calculated for each individual time step. Due to the complete exchange of the acceptor buffer at each time step, $c_{A,tx-1}$ was always 0.

The calculated P_{app} were corrected for recovery according to Neuhoff (2005):

$$\boldsymbol{P_{app,corr.}} = \frac{\boldsymbol{P_{app}} \cdot 100}{\boldsymbol{Recovery}} \tag{14}$$

If not stated otherwise, P_{app} refers to the recovery-corrected version in the following sections.

In order to obtain the mean P_{app} and the associated standard deviation, P_{app} from the 10–20 min and the 20–30 min time interval of both replicates were averaged. In order to account for the lag time, we decided to not include the 0–10 min time interval in the calculation of the mean P_{app} (Heikkinen et al., 2009).

3.2.4. Assessment of monolayer integrity

In order to assess the integrity of the MDCK monolayers, the a→b transport of the paracellular marker Lucifer Yellow was determined at pH 7.4→7.4. After each transport experiment, the inserts were moved to new well plates containing 1.6 ml HBSS 7.4 in the basolateral acceptor compartment. The apical volume was discarded and replaced by 0.5 ml Lucifer Yellow stock solution (100 μg/ml). The well plates were placed on an orbital shaker set to 37 °C and 450 rpm. After 60 min, samples were taken from the basolateral acceptor compartment and the fluorescence intensity (Ex: 485 nm/Em: 538 nm) was analyzed with a Tecan Infinite M1000 Pro plate reader (Tecan Group Ltd., Männedorf, Switzerland). Inserts were excluded if the P_{app} of Lucifer Yellow exceeded 1×10^{-6} cm/s. The average LY permeability was $3.4 \pm 1 \times 10^{-7}$ cm/s. Only 3 out of 130 inserts had to be excluded due to LY permeabilities larger than 1×10^{-6} cm/s.

In addition, the TEER of each insert was measured at three different positions immediately before and after the transport experiments with an EVOM epithelial voltohmmeter (World Precision Instruments Inc., Sarasota, USA). The average TEER of the inserts was 140 ± 5 Ω cm^2 before and 138 ± 7 Ω cm^2 after the transport experiments, independent of the pH conditions, thereby confirming the integrity of the cell monolayers throughout the experiment.

3.3. Determination of cytosolic pH

In order to determine the pH_{cyt} of MDCK cells, the pH-sensitive dye pHrodo Red was used in three different scenarios, where mainly dye concentration and plate format was varied. The differences are described in detail in Table S1 in the Supporting Material.

In contrast to the transport experiments, the cells were seeded directly into the well plates, not on PET inserts. One well plate was used for the determination of pH_{cyt} and one well plate was used for the intracellular calibration. The cells were maintained as described in Section 3.1. After growing to confluency, the cell monolayers were washed twice with HBSS pH 7.4 to remove residual DMEM and incubated for 30 min with pHrodo Red AM dissolved in HBSS pH 7.4. In order to facilitate the cellular uptake of pHrodo Red AM, 1 % PowerLoad concentrate was added to the dye solution. After the incubation period, the cells were washed twice with HBSS pH 7.4 to remove residual dye.

For determination of pH_{cyt}, the cells were exposed to either HBSS pH 5, 5.5, 6, 6.5, 7, 7.4 or 8. The fluorescence intensity (Ex: 560 nm/Em: 580 nm) was measured at regular time steps. The fluorescence intensity of dye-free control cells was subtracted to correct for background fluorescence.

For intracellular calibration, the nigericin technique introduced by Thomas et al. (1979) was used: 10 μM nigericin and 135 mM KCl were added to HBSS pH 5, 5.5, 6, 6.5, 7, 7.4 and 8. The cells were incubated with the calibration buffers for 10 min. The fluorescence intensity (Ex: 560 nm/Em: 580 nm) was measured and plotted against the pH of the calibration buffers to obtain the calibration curve.

All chemicals used in this study are listed in Table S2 in the Supporting Material.

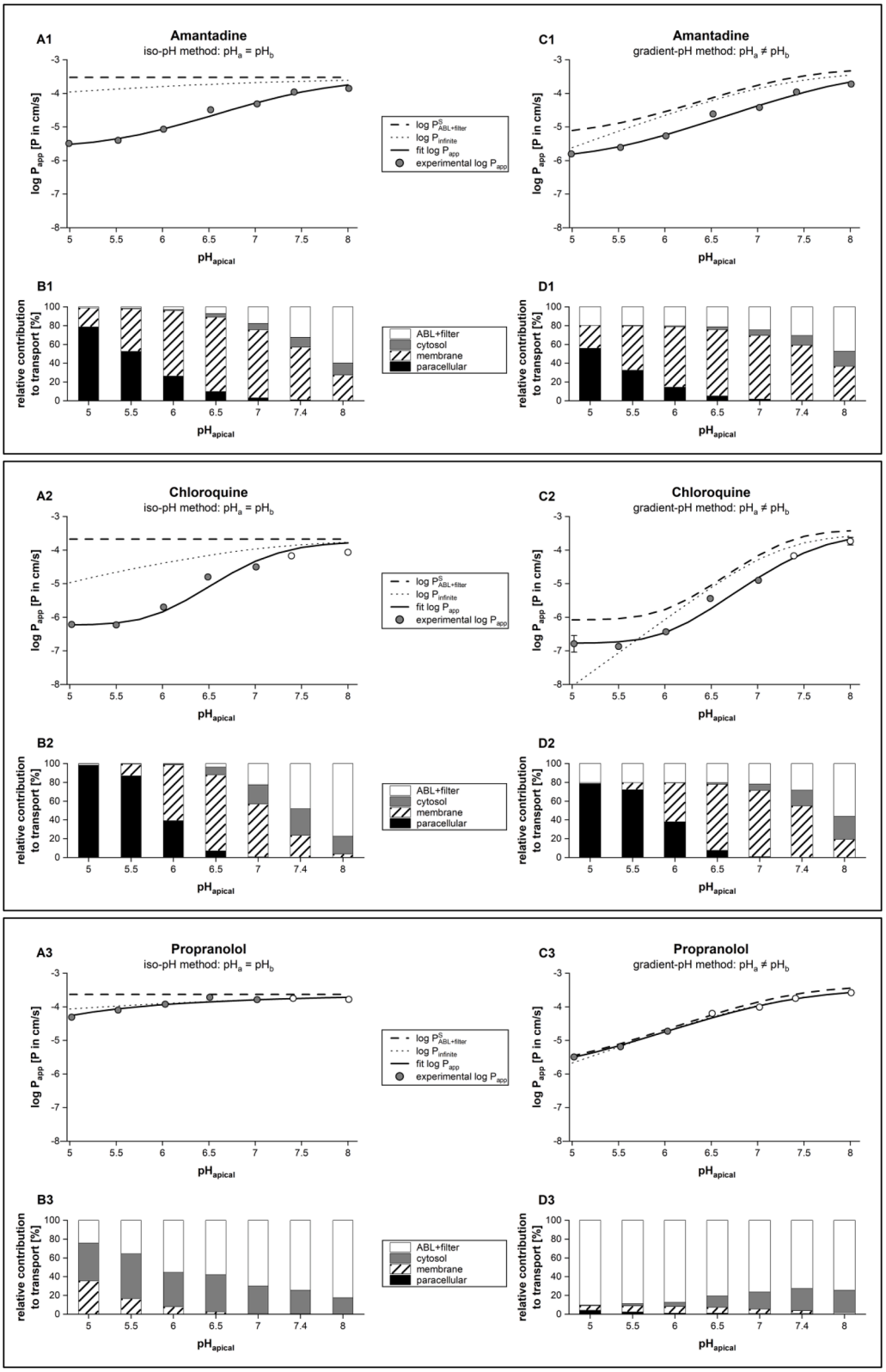

Fig. 2. Comparison of apparent permeability (P_{app}) and composition of P_{app} in the iso-pH method and the gradient-pH method. (A,C) Log P_{app} determined as a function of apical pH in MDCK transwell assays. The circles represent experimental log P_{app} corrected for recovery. All values are listed in Table S4 in the Supporting Material. Standard deviations are only shown where they exceed the size of the symbol. Filled circles indicate recoveries larger than 80 % and were included in the fit. Unfilled circles indicate recoveries less than 80 % and were not included in the fit. The fits were performed using Igor Pro 7 and are shown as solid lines. The dashed line represents the log permeability of ABL and filter (log $P^{S}_{ABL+filter}$). The dotted line represents the theoretical curve for log P_{app} assuming an infinitely high P_0 (log $P_{infinite}$). (B,D) Relative contributions of the individual permeation barriers ABL+filter, cytosol, membrane and paracellular to the total permeation process at different apical pH values.

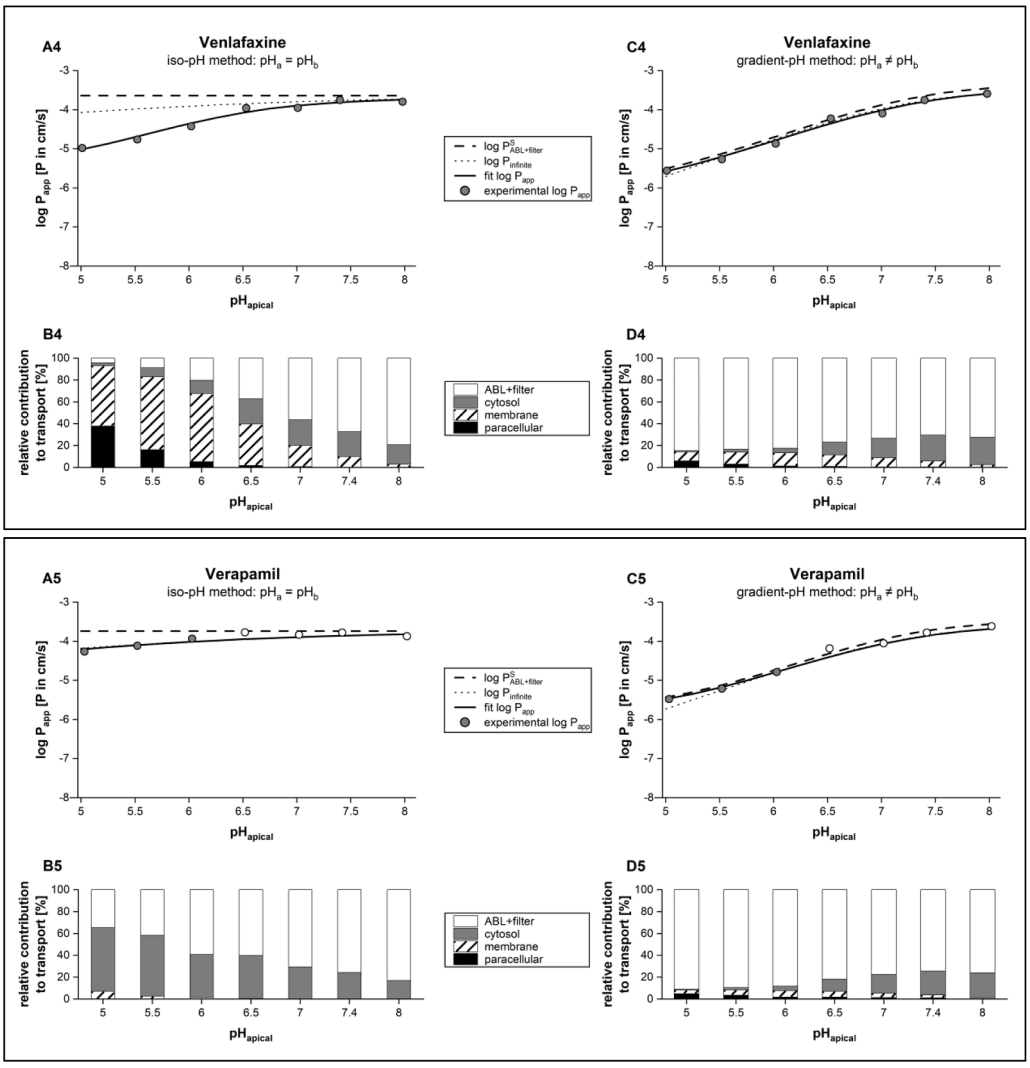

Fig. 2. (*continued*).

4. Results and discussion

4.1. Extraction of the intrinsic membrane permeability from gradient-pH and iso-pH experiments

The P_{app} of the five lipophilic bases amantadine, chloroquine, propranolol, venlafaxine and verapamil was measured in transwell experiments using the iso-pH method and the gradient-pH method. The resulting recovery-corrected log P_{app} (see Tables S3 and S4 in the Supporting Material) are plotted against pH_a in Fig. 2A1–A5 and C1–C5, respectively. For all compounds a substantial difference between the log P_{app} measured with the gradient-pH method and the log P_{app} measured with the iso-pH method is evident. However, all graphs show a diagonal section which is assumed to indicate a substantial contribution of P_m to P_{app} (Lozoya-Agullo et al., 2017). Consequently, the extraction of P_0 should be possible from each experiment. In the following, we will show that this is not true.

In order to extract the P_0, fits according to Eq. (4) were performed for each compound and each method using Igor Pro 7 (WaveMetrics Inc., Lake Oswego, USA). According to Eq. (5), all P_0 values were determined using a factor of 24 to account for the increased surface area of the apical membrane due to microvilli. This factor might vary between different cell types (Meng et al., 2017). For comparison, we therefore performed additional fits using a factor of 1 instead of 24. The extracted P_0 differ by less than a factor of 2 (see Eqs. (S15)–(S17) and Table S5 in the Supporting Material). Data points were only included in the fit if the recovery exceeded 80 % (indicated by filled circles). P_0 and P_{para} were set as free parameters while P_{cyt}, P_{filter}, x_a, x_b, pK_a and D_w were set as fixed parameters. Details about the fit and fit parameters can be found in Fig. S3 and Tables S6–S10 in the Supporting Material. The fits are shown as solid lines in Fig. 2A1–A5 and C1–C5, respectively. For all compounds, the log P_{app} fit curve is well-matched to all data points, even the ones that were not included in the fit due to low recoveries (indicated by unfilled circles). It is noticeable that the experimental log P_{app} at pH 6.5 is systematically higher than the fitted log P_{app}. This can be attributed to the lower pH stability of the pH 6.5 buffer compared to the other buffers (see Section 3.2.2).

In addition to the solid log P_{app} fit curves, each figure includes a dashed curve representing log $P^S_{ABL+filter}$. This curve represents the calculated permeability of ABL and filter, using the concentration-shift factors extracted from the fit. It illustrates the main difference between the iso-pH method and the gradient-pH method. If the iso-pH method is used, pH_a and pH_b are identical. Consequently, no concentration-shift effect occurs in the basolateral ABL and the filter, and log $P^S_{ABL+filter}$ remains constant, independent of pH (see Fig. 2A1-A5). If the gradient-pH method is used, a concentration-shift effect occurs in the basolateral ABL and filter due to the applied pH-difference. For bases, this concentration-shift effect is negative for pH_a values below 7.4, causing log $P^S_{ABL+filter}$ to decrease, and positive for pH_a values

above 7.4, causing log $P^S_{ABL+filter}$ to increase. Consequently, log $P^S_{ABL+filter}$ is not constant in the gradient-pH method, but a function of pH_a (see Fig. 2C1–C5). In this aspect, the method presented in this work differs significantly from the previously used method, in which $P^S_{ABL+filter}$ was considered pH-independent in the gradient-pH method, since concentration-shift factors were not taken into account. For amantadine (see Fig. 2C1) and chloroquine (see Fig. 2C2), a flattening of the log $P^S_{ABL+filter}$ curve towards a lower plateau can be observed at low pH_a. Due to the low P_m of amantadine and chloroquine at low pH_a, the a→b transport of both compounds is dominated by the paracellular route rather than the transcellular route at low pH_a. A negligibly small $P_{trans,\ a\rightarrow b}$ results in a constant concentration-shift factor (see Eq. (7)) and therefore a constant $P^S_{ABL+filter}$ plateau at low pH_a.

In order to determine a reliable P_0, there must be a substantial contribution of the membrane to the total permeation resistance. In order to assess whether the log P_{app} fit curve contains a substantial contribution from the membrane or is simply a result of the aqueous barriers (ABL, filter and cytosol), we introduced a third dotted curve. For this curve we assumed P_0 to be infinitely high. For this reason, P_0 was set to 10,000 cm/s in the fit equation. The resulting log $P_{infinite}$ curve differs from the log $P^S_{ABL+filter}$ curve in two aspects: First, the cytosol, together with the associated concentration-shift effects, is considered as an additional aqueous barrier. Second, due to the infinitely high P_0, P_{para} is negligibly small. According to Eq. (7), this means that the concentration-shift factors in the basolateral ABL and filter are at their respective maximum at all pH. Therefore, in contrast to the log $P^S_{ABL+filter}$ curve, there is no flattening of the log $P_{infinite}$ curve towards a lower plateau. The dominance of ABL, filter and cytosol can be excluded if the log P_{app} fit curve is substantially different from the log $P_{infinite}$ curve. In order to extract P_0, P_{app} must differ not only from ABL, filter and cytosol, but also from P_{para}. In order to visualize the contribution of the membrane to the total permeation process, the relative contributions of the different permeation sub-processes are shown in Fig. 2B1–B5 and D1-D5 for each individual data point.

For amantadine and chloroquine, the log $P_{infinite}$ curve is substantially different from the log P_{app} fit curve in both the iso-pH method and the gradient-pH method. This indicates that the permeation process is not exclusively dominated by ABL, filter and cytosol. At low pH_a, the paracellular transport is the dominating sub-process (see Fig. 2B1, B2 and D1, D2). But at medium pH_a, the membrane is the dominating resistance and consequently P_0 can be extracted. For amantadine a log P_0 of −0.84 was determined from the iso-pH method fit curve and a log P_0 of −0.88 was determined from the gradient-pH method fit curve. The fact that both values are almost identical shows that P_0 can in principle be extracted from both methods, if occurring concentration-shift effects are considered correctly. For chloroquine a log P_0 of 0.05 was determined from the iso-pH method fit curve and a log P_0 of −0.54 was determined from the gradient-pH method fit curve. Unlike amantadine, both P_0 are not identical, but differ by a factor of 4. This difference in fitted P_0 explains why the pH 7.4 bars in Fig. 2B2 and D2 are not identical, although they are based on the same P_{app} measurement. There is also a substantial difference between the two methods in P_{para}. Compared to the gradient-pH method, P_{para} is larger by a factor of 3 when the iso-pH method is used. This might indicate that a pH-difference has additional effects on dications such as chloroquine compared to monocations such as amantadine.

For venlafaxine the log $P_{infinite}$ and the log P_{app} fit curve are congruent, when the gradient-pH method is used (see Fig. 2C4). This indicates that the permeation process is dominated by ABL, filter and cytosol and the contribution of the membrane to the total permeation process is minimal at any pH (see Fig. 2D4). Consequently, no P_0 can be extracted from the gradient-pH data. Note that the contribution of the membrane to the total permeation process depicted in Fig. 2D4 is not reliable, since it only reflects the unreliable P_0 value that resulted from the fit. This example clearly shows that, contrary to the previous assumption, a diagonal section in the gradient-pH plot does not necessarily represent a substantial contribution of the membrane to the total permeation process. The diagonal section can simply be a result of the pH-dependence of ABL, filter and cytosol permeability. By using the iso-pH method, concentration-shift effects in the basolateral ABL and filter, leading to increased ABL and filter resistance, are avoided. A substantial difference between the log $P_{infinite}$ and the log P_{app} fit curve can be observed at low pH_a (see Fig. 2A4). The contribution of the paracellular transport is rather small. Consequently, the permeation process is dominated by the membrane at low pH_a (see Fig. 2B4) and a reliable log P_0 of −0.83 can be extracted from the iso-pH method fit curve. Thus, venlafaxine is a good example of why avoiding concentration-shift effects in the basolateral ABL and filter by using the iso-pH method is beneficial for the extraction of the P_0 of lipophilic bases.

Similar to venlafaxine, the log $P_{infinite}$ and the log P_{app} fit curve of propranolol (see Fig. 2C3) and verapamil (see Fig. 2C5) are congruent, when the gradient-pH method is used. Consequently, no P_0 can be extracted. Unlike venlafaxine, using the iso-pH method does not result in a substantial difference between log $P_{infinite}$ and log P_{app} fit curve either. For propranolol, the log P_{app} fit curve slightly differs from the log $P_{infinite}$ curve at low pH_a, which might indicate a contribution of the membrane to the overall permeation process. However, since P_{app} is predominantly determined by the cytosol, it might also be just a result of the uncertainties in pH_{cyt}. Hence, for these two compounds no reliable P_0 can be extracted from the iso-pH data either. Therefore, only lower limits for log P_0 were determined for propranolol (log $P_0 \gtrapprox 0.02$) and verapamil (log $P_0 \gtrapprox 0.07$) (see Eq. (S18) in the Supporting Material). The relative contribution of the membrane might thus be even lower than shown in Fig. 2B3 and B5. The examples of propranolol and verapamil show that a diagonal section in the iso-pH plot does also not necessarily represent a substantial contribution of the membrane to the total permeation process. When the iso-pH method is used, there is no concentration-shift effect in ABL and filter, but there is a concentration-shift effect in the cytosol, that needs to be considered. For bases, the concentration-shift factor of the cytosol decreases with decreasing pH_a. Consequently, the resistance of the cytosol increases and can become the dominating resistance. The diagonal section in the iso-pH plot of propranolol and verapamil is a consequence of this increasing cytosol resistance. In this aspect, the method presented in this work differs significantly from the previously used method, in which the resistance of the cytosol is assumed to be negligible, since concentration-shift factors were not taken into account.

For lipophilic bases, it can be summarized that, contrary to the previous assumption, a diagonal section in the log P_{app} vs pH_a plot does not necessarily represent a substantial contribution of the membrane to the total permeation process. The diagonal section in the gradient-pH plot can be a result of the pH-dependence of $P^S_{ABL+filter}$. The diagonal section in the iso-pH plot can be a result of the pH-dependence of P^S_{cyt}. In principle, P_0 can be extracted from both, the gradient-pH as well as the iso-pH method if concentration-shift effects are considered correctly. Nevertheless, the iso-pH method has proven to be beneficial for the extraction of P_0 of lipophilic bases, as an increase in ABL and filter resistance due to negative concentration-shift effects can be avoided. However, even when using the iso-pH method, there are still compounds for which no reliable P_0 can be determined, but only a lower limit for P_0.

4.2. Comparison of own data and literature data

In order to demonstrate the importance of considering the influence of aqueous barriers such as the ABL, filter and cytosol and the associated concentration-shift effects, the log P_0 values determined in this work are compared to log P_0 values reported in literature (Avdeef, 2012) in Table 1.

For propranolol and verapamil gradient-pH data are available from Avdeef et al. (2005). Based on these data, a log P_0 of −1.76 was

Table 1
Comparison of log P_0 determined in this work by the iso-pH method and log P_0 reported in literature.

Compound	Log P_0 [P in cm/s]	
	This work [a]	Literature [b]
Amantadine	−0.84	−2.17
Chloroquine	0.05	−1.18
Propranolol	≳ 0.02	−1.76
Venlafaxine	−0.83	−2.84
Verapamil	≳ 0.07	−2.45

[a] log P_0 extracted according to Eq. (4) from log P_{app} determined in this work by the iso-pH method.
[b] log P_0 extracted by Avdeef (2012) from log P_{app} determined by Summerfield et al. (2007) (amantadine, venlafaxine); Richter et al. (2009) and Hayeshi et al. (2006) (chloroquine) or Avdeef et al. (2005) (propranolol, verapamil).

determined for propranolol and a log P_0 of −2.45 was determined for verapamil (Avdeef, 2012). As discussed in Section 4.1, extracting a reliable P_0 for propranolol and verapamil from gradient-pH data is not possible, even if concentration-shift effects in the basolateral ABL and filter are considered correctly. In literature, these concentration-shift effects have not been considered and the diagonal section in the gradient-pH plots has been misinterpreted as a substantial contribution of the membrane to the total permeation process. Consequently, the published log P_0 for propranolol and verapamil are substantially too low.

Regarding the log P_{app} data of propranolol and verapamil, there is one noticeable difference between the log P_{app} reported by Avdeef et al. (2005) and the log P_{app} determined in this work. While at high pH_a the log P_{app} values are in good agreement, at low pH_a, a plateau appears in Avdeef's data, which could not be reproduced in this work (see Fig. S4 in the Supporting Material). According to Avdeef (2012) this lower plateau in the gradient-pH plot is about two log units higher than the expected log P_{para}. It has been assumed that this unexpectedly high plateau either represents the membrane permeability of the cation (P_{cation}) or, more likely, indicates the involvement of influx transporters (Avdeef et al., 2005; Avdeef, 2012). P_{ion} is usually much lower than $P_{neutral}$ due to the Born energy and P_{cation} is even smaller than P_{anion} due to the positive dipole potential of membranes (Schwöbel et al., 2020). Our estimates (see Eq. (S19) in the Supporting Material) indicate that log P_{cation} of propranolol and verapamil is lower than −11 and thus substantially lower than log P_{para}. The unexpectedly high lower plateau can therefore not be attributed to P_{cation}, but possibly to active transport. This might explain why we were not able to reproduce this plateau in our own experiments or calculations. It is possible that the MDCK cells used in our experiments do not express the same influx transporters as the Caco-2 cells used in Avdeef's experiments or that these influx transporters are inhibited by elacridar in our experiments. Another possibility is that P_{para} is substantially higher than expected. The estimation of P_{para} is usually optimized for hydrophilic compounds (Avdeef, 2010) and might therefore not be suitable to lipophilic compounds such as propranolol or verapamil.

For amantadine, chloroquine and venlafaxine, no pH-dependent measurement of P_{app} is available from the literature for the extraction of P_0. Instead, the extraction of P_0 is based on a single measurement of P_{app} at pH 7.4→7.4. Given that no concentration-shift effects in basolateral ABL and filter need to be considered, when the P_{app} is measured at pH 7.4→7.4, one could assume that the log P_0 reported in literature for amantadine, venlafaxine and chloroquine (Avdeef, 2012) should fit our own experimental data.

The log P_0 of amantadine and venlafaxine are extracted from Summerfield et al. (2007) who measured the P_{app} in MDCK-MDR1 cells using elacridar to inhibit active transport. For amantadine a log P_{app} of -5.15 (P_{app} of 7.1×10^{-6} cm/s) and for venlafaxine a log P_{app} of −4.93 (P_{app} of 11.7×10^{-6} cm/s) was determined. In our own experiments, we determined a log P_{app} of −3.96 for amantadine and −3.75 for venlafaxine at pH 7.4→7.4. A possible reason for the differences in P_{app} is the applied shaking speed. In our experiments we used a shaking speed of 450 rpm, while Summerfield et al. (2007) used a substantially lower shaking speed of 150 rpm. The lower the shaking speed the larger the ABL thickness and consequently the lower the ABL permeability (Karlsson and Artursson, 1991). The relative contribution of the ABL to the total permeation process increases and consequently the relative contribution of the membrane decreases. In order to evaluate the contributions of the different sub-processes a pH-dependent measurement of P_{app} would have been desirable. But since no pH-dependent measurement was carried out by Summerfield et al. (2007), we can only hypothesize that the P_{app} of amantadine and venlafaxine at pH 7.4 was dominated by the ABL. It is therefore not surprising that the log P_0 extracted from this P_{app} by Avdeef (2012) is substantially lower than the log P_0 determined in this work. However, it needs to be noted that the discrepancy between the P_{app} in this work and the P_{app} reported in literature cannot be attributed exclusively to an increased ABL thickness, as the ABL thickness would be unrealistically large. It can therefore be assumed that other problems, such as low recoveries, also contributed to the low P_{app} in the Summerfield dataset.

The log P_0 of chloroquine is extracted from the P_{app} determined by Richter et al. (2009) who used Caco-2 and MDCK-II cells and a shaking speed of 100 rpm and Hayeshi et al. (2006) who used Caco-2 cells and a shaking speed of 450 rpm. Richter et al. (2009) determined log P_{app} of −4.62 (P_{app} of 24×10^{-6}) for MDCK-II and −5.06 (P_{app} of 8.68×10^{-6}) for Caco-2 cells. Similar to amantadine and venlafaxine, the difference to the log P_{app} of −4.17 determined in this work, might be explained by the low shaking rate and the resulting large ABL thickness. Hayeshi et al. (2006) measured a log P_{app} of −4.25 to −3.94 (P_{app} of 56×10^{-6} to 116×10^{-6}) depending on the chloroquine concentration. This is in good agreement with the P_{app} of −4.17 determined in this work. Although there is a good agreement between both values, Fig. 2B2 shows that the contribution of the membrane to the total permeation process is not very large at pH 7.4. In addition, the recovery of chloroquine at pH 7.4 is only 55 %. Therefore, this data point was not included in our fit. Both aspects lead to uncertainties in the extracted log P_0 from the Hayeshi data and might explain the difference from the log P_0 extracted from multiple pH values in this work.

For all lipophilic bases, the log P_0 extracted from our own experiments was at least one order of magnitude larger than the log P_0 reported in literature. This finding is supported by Korjamo et al. (2008), who also found that the P_0 extracted from iso-pH data is substantially higher than the published P_0 extracted from gradient-pH data. The underestimation of P_0 is a consequence of the underestimation of ABL effects. In order to minimize ABL effects, the transport experiments should be carried out at an appropriate shaking speed. Differing pH values between apical and basolateral compartment should be avoided. For lipophilic bases, it is advantageous to use pH values different from 7.4. At high pH, lipophilic bases are predominantly uncharged which leads to high membrane permeabilities. Consequently, the ABL, not the membrane, is the limiting resistance in the permeation process and extraction of P_0 is not possible. In order to extract P_0 from the measured P_{app}, it is therefore necessary to lower the pH to reduce the neutral fraction and therefore the membrane permeability of the lipophilic base. Most information about all involved sub-processes is gained by measuring the P_{app} at various pH values.

4.3. Re-evaluation of gradient-pH data from literature

Having successfully applied the revised method for the extraction of P_0 to our own experimental dataset, we applied it to gradient-pH data reported in literature. Similar to our own dataset, we performed a fit according to Eq. (4) for each individual compound. The fits and fit parameters are shown in Fig. S5 in the Supporting Material. The resulting log P_0 are presented in Table 2 and are compared to log P_0 determined by the original gradient-pH method, which does not consider

Table 2
Comparison of log P_0 determined by the revisited gradient-pH method which considers concentration-shift effects, and the original gradient-pH method which does not consider concentration-shift effects in the data evaluation.

Compound	Log P_0 [P in cm/s]	
	Revisited gradient-pH method [a]	Original gradient-pH method [b]
Alfentanil	$\gtrapprox -2.87$	−3.46
Atenolol	−4.38	−4.45
Cimetidine	−6.13	−6.12
Indomethacin	−0.21	−0.84
Metoprolol	−1.35	−1.79
Salicylic acid	−0.20	−0.43

[a] log P_0 extracted according to Eq. (4) from log P_{app} determined by Nagahara et al. (2004) (alfentanil, cimetidine); Neuhoff et al. (2003) (atenolol, metoprolol) or Neuhoff et al. (2005) (indomethacin, salicylic acid).

[b] log P_0 extracted by Avdeef et al. (2012) from log P_{app} determined by Nagahara et al. (2004) (alfentanil, cimetidine); Neuhoff et al. (2003) (atenolol, metoprolol); Neuhoff et al. (2005) (indomethacin) or Karlsson and Artursson (1991), Yamashita et al. (2000), Irvine et al. (1999) and Neuhoff et al. (2005) (salicylic acid).

concentration-shift effects.

For the lipophilic base alfentanil (log $K_{hex/w}$: −0.71), the log P_{app} fit curve and the log $P_{infinite}$ curve are congruent. This implies that the membrane resistance does not contribute substantially to the total resistance. Therefore, only a lower limit for log P_0 could be determined from the gradient-pH data. When the iso-pH method is used, extraction of a reliable log P_0 might be possible, or at least a lower limit closer to the actual log P_0 can be determined.

For the lipophilic base metoprolol (log $K_{hex/w}$: −0.44) and the lipophilic acid indomethacin (log $K_{hex/w}$: 0.92), the log P_{app} fit curve and the log $P_{infinite}$ curve are not congruent. However, the differences between both curves do not exceed a factor of 2 at any pH. Since the fit is based on many datapoints and the standard deviations of these datapoints are very small, a log P_0 can be extracted for both compounds, but it must be considered uncertain. The iso-pH method should be used to confirm the log P_0. For bases such as metoprolol, the iso-pH method is clearly advantageous at pH values below 7.4 as no negative concentration-shift effects occur that increase ABL and filter resistance. For metoprolol, iso-pH data from Korjamo et al. (2008) are available, confirming a log P_0 of −1.40 (see Fig. S5 in the Supporting Material). In contrast, positive concentration-shift effects that decrease ABL and filter resistance occur with acids at pH values below 7.4 when the gradient-pH method is used. So why should the iso-pH method be used for acids such as indomethacin? Due to the fact that with indomethacin, ABL and filter are the dominating resistances at pH values below 7.4 despite the positive concentration-shift effect. In order to extract the log P_0, a pH above 7.4 is required. At pH values above 7.4 negative concentration-shift effects occur with acids when the gradient-pH method is used. In order to avoid these negative concentration-shift effects, the iso-pH method should be used. Unfortunately, iso-pH data for indomethacin are only available for pH 5, 6.5 and 7.4 (Neuhoff et al., 2005). In order to confirm the log P_0 of −0.21, the P_{app} of indomethacin should be measured at pH 8. Nevertheless, it needs to be noted that even at pH 8 the difference between the log P_{app} fit curve and the log $P_{infinite}$ curve would barely exceed a factor of 2.

For the lipophilic acid salicylic acid (log $K_{hex/w}$: −0.72) there is a substantial difference between the log P_{app} fit curve and the log $P_{infinite}$ curve, which implies a substantial contribution of the membrane resistance to the total resistance. Thus, a reliable log P_0 can be extracted from the gradient-pH data. This log P_0 is confirmed by the log P_0 of −0.39 that can be extracted from the iso-pH data available from Neuhoff et al. (2005) (see Fig. S5 in the Supporting Material).

For the hydrophilic bases atenolol (log $K_{hex/w}$: −4.55) and cimetidine (log $K_{hex/w}$: −4.7) the aqueous barriers (ABL, cytosol and filter) contribute almost nothing to the total resistance due to the very low membrane permeability of both compounds. Consequently, there is a large difference between the log P_{app} fit curve and the log $P_{infinite}$ curve. In order to extract a reliable log P_0 for hydrophilic compounds, it is more important that the log P_{app} fit curve is substantially different from log P_{para}. This was the case with atenolol and cimetidine and consequently the extraction of a reliable log P_0 was possible.

Compared to the log P_0 reported in literature, the consideration of concentration-shift effects results in a substantially higher log P_0 for all compounds except atenolol und cimetidine. Atenolol and cimetidine are exceptions, as ABL and filter resistance and the associated concentration-shift factors are negligible due to the very low membrane permeability of these compounds. However, if ABL and filter contribute to the permeation process, concentration-shift effects need to be considered when the gradient-pH method is used.

4.4. Thickness of apical and basolateral ABL

The thickness of the ABL in Caco-2 or MDCK transwell experiments is influenced by the experimental setup and the applied shaking speed. The total ABL thickness can be determined by ABL markers such as testosterone (Karlsson and Artursson, 1991). But to correctly determine the concentration-shift effect in the basolateral ABL, knowing the total ABL thickness is not enough. The thicknesses of the apical and basolateral ABL need to be determined separately. For this reason, we conducted a global fit according to Eq. (4) using Igor Pro 7 Global Fit Package. In this global fit, all recovery-corrected log P_{app} values from the iso-pH and the gradient-pH method of amantadine and venlafaxine were included and weighted by standard deviations. Chloroquine, propranolol and verapamil were not included in the global fit, because, unlike amantadine and venlafaxine, these compounds do not have constant recoveries above 80 %. Given that the D_w of amantadine, chloroquine, propranolol, venlafaxine and verapamil are very similar (6.3×10^{-6} to 10.3×10^{-6} cm^2/s), similar ABL thicknesses are expected for all compounds (Pohl et al., 1998). Using a shaking speed of 450 rpm, we determined an apical ABL thickness of 176 μm and a basolateral ABL thickness of 75 μm for our setup. Asymmetric ABL thicknesses in transwell experiments have also been reported by Korjamo et al. (2008). Using a shaking speed of 420 rpm, they reported apical ABL thicknesses ranging between 160 μm and 300 μm and basolateral ABL thicknesses ranging between 37 μm and 130 μm (Korjamo et al., 2008). Similar to our results, the thickness of the basolateral ABL was substantially lower than the thickness of the apical ABL in each case.

4.5. Cytosolic pH

In order to quantify the concentration-shift effects in the cytosol according to Eq. (6) reliable pH_{cyt} values are needed. For this reason, we measured the pH_{cyt} of MDCK cells as a function of external pH_e. Table 3 shows the average pH_{cyt} of three independent measurements after an incubation period of 10 min.

Contrary to expectations, the pH_{cyt} of MDCK cells at a pH_e of 7.4 is 7.7, not 7.4. Already after a short incubation period of 10 min, the pH_{cyt} seems to adapt to the pH_e. This trend was also observed by Chatton and

Table 3
Effect of the external pH (pH_e) on the cytosolic pH (pH_{cyt}) of MDCK cells after a 10 min incubation period.

pH_e	pH_{cyt}
5.0	6.0 ± 0.22
5.5	6.4 ± 0.13
6.0	6.6 ± 0.10
6.5	7.1 ± 0.02
7.0	7.4 ± 0.07
7.4	7.7 ± 0.10
8.0	8.1 ± 0.28

Spring (1994) and Borle and Bender (1991) for MDCK cells and Michl et al. (2019), Liang et al. (2007) and Perdikis et al. (1998) for Caco-2 cells. However, comparing the absolute pH_{cyt} values is difficult as they depend on the cell type and buffer system (Michl et al., 2019) as well as on the selected method and incubation time. An overview of the experimental pH_{cyt} reported in literature and the change of pH_{cyt} over time is provided in Figs. S6 and S7 in the Supporting Material. Our results clearly rebut the assumption that the pH_{cyt} of MDCK cells in *in vitro* assays is maintained constant at a pH of 7.4 independent of pH_e. Compared to a constant pH_{cyt} of 7.4, the difference between actual pH_{cyt} and pH_e is substantially smaller (with the exception of pH_e 7.4). Consequently, the concentration-shift effect in the cytosol is less pronounced than expected if a constant pH of 7.4 in the cytosol is assumed. Since the pH_{cyt} in our experiment is consistently higher than the pH_e, the concentration-shift effect in the cytosol is always negative for bases and always positive for acids (see Fig. S2B in the Supporting Material).

It needs to be noted that the determined pH_{cyt} values are only applicable to the iso-pH method, as our experimental setup did not allow applying a pH-difference for the measurement of pH_{cyt}. It can be hypothesized that the pH_{cyt} remains closer to the initial pH of 7.7 if only the apical pH is varied and the basolateral pH is maintained constant at 7.4. However, since the concentration-shift effect in basolateral ABL and filter in the gradient-pH method clearly outweighs the concentration-shift effect in the cytosol, knowledge of the exact pH_{cyt} is less relevant in the gradient-pH method.

5. Conclusion

In this study, we demonstrated experimentally and theoretically that P_{app} and the contributions of the individual permeation barriers to P_{app} differ substantially between iso-pH and gradient-pH method. These differences are caused by concentration-shift effects in the basolateral ABL and filter occurring in the gradient-pH method due to the applied pH-difference between apical and basolateral compartment. In addition, concentration-shift effects in the cytosol occur regardless of the method due to the pH-difference between apical compartment and cytosol. In the original gradient-pH method, these concentration-shift effects were not considered in the extraction of P_0. In many cases, the extracted P_0 are therefore substantially too low. If the concentration-shift effects are considered, it is in principle possible to extract reliable P_0 from both the gradient-pH and the iso-pH method, since P_0 itself is not affected by concentration-shift effects. Which method is more advantageous depends on the compound and the pH range of interest. For lipophilic bases, the iso-pH method has proven to be advantageous because, in contrast to the gradient-pH method, negative concentration-shift effects at pH values below 7.4 are avoided. However, for some compounds, extraction of a reliable P_0 is neither possible from the iso-pH method nor from the gradient-pH method due to their very high membrane permeability. For these compounds, only lower limits can be determined for P_0.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2023.106592.

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