Mimicking regional and fasted/fed state conditions in the intestine with the mucus-PVPA *in vitro* model: the impact of pH and simulated intestinal fluids on drug permeability

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Abstract

Intestinal drug absorption following oral administration can be influenced by regional conditions (absorbing surface area, bacterial flora, motility, pH, mucus thickness) and food intake, all of which affect drug solubility and permeability. Therefore, it is crucial to assess the impact of these conditions on the drugability of drugs and formulations. In this study, the ability of the liposome-based mucus-PVPA *in vitro* permeability model to handle relevant intestinal pH conditions was evaluated, together with the investigation on the pH-dependent solubility and permeability profiles of five model drugs. This study additionally evaluated the impact of all commercially available versions of the fasted and fed state simulated intestinal fluids (SIFs) on the integrity of the barriers, and the permeabilities of one hydrophilic and one lipophilic compound were examined under these conditions. The model was found to be well-functioning in all tested pH conditions, and a pH-dependent trend was found for both solubility and permeability profiles for acidic and basic compounds, according to their degree of ionisation. Moreover, the mucus layer and its pH-dependent viscosity particularly influenced the permeation of more lipophilic compounds. The PVPA barriers primarily maintained their functionality in the presence of the fed state SIFs, and the permeability of the two tested compounds showed to be influenced by their hydrophilicity/lipophilicity, their degree of interaction with mucus and by the bile salts and phospholipids content in the SIFs. Overall, the obtained results highlight the relevance of studying the effect that pH, mucus and SIFs have on intestinal drug absorption, and suggest the suitability of the mucus-PVPA model for such investigations.

Keywords

In vitro model; pH-dependent permeability; pH-dependent solubility; mucus; FaSSIF/FeSSIF; simulated intestinal fluids

1. Introduction

The small intestine forms the largest part of the gastrointestinal (GI) tract promoting the absorption of orally administered drugs (Billat et al., 2017). Its three segments (namely, duodenum, jejunum and ileum) are characterized by differences in length, absorbing surface area, bacterial flora, motility, pH and mucus thickness (Billat et al., 2017). The different regional characteristics can influence the solubility and permeability of drugs, and thereby their absorption after oral administration. For instance, the changes in pH through the length of the GI tract can influence the ionisation of the drugs and thus their intestinal absorption, as suggested by the pH partition hypothesis (Shore et al., 1957). Changes in pH can also affect the hydrophilic mucus layer, which lines, lubricates and protects the GI tract. This layer is the first barrier that drugs need to overcome in order to explicate their effect (Johansson et al., 2013), and changes in pH can affect its structure and rheology, consequently impacting the diffusion properties of the drugs through it (Cao et al., 1999; Lieleg et al., 2010).
In addition to the regional physiological changes in the GI tract, the characteristics and composition of the intestinal fluids vary widely according to the pre- or post- prandial state (Clarysse et al., 2009; Riethorst et al., 2016; Riethorst et al., 2018). In this regard, it has been demonstrated that bile salts and phospholipids in the human intestinal fluids can affect drug solubilisation, and thus influence permeability through the intestinal walls (Riethorst et al., 2018). These regional and nutritional differences can also have an effect on drug absorption in a different manner according to the intrinsic characteristics of the drug in consideration and to its formulation features (Augustijns et al., 2014). A conspicuous effort has thus been put into simulating the human intestinal fluids, and as a result different versions of fasted and fed state simulated intestinal fluids (FaSSIF and FeSSIF) have been proposed (Galia et al., 1998; Jantratid et al., 2008).

Since all these variables can affect the absorption of drugs and formulations, understanding their impact is crucial, especially as oral drug administration is still regarded as the leading route for drug delivery due to its accessibility, great patient compliance and cost-effectiveness (Berben et al., 2018a). To assess the impact of these variables on drugability (i.e. the ability of a drug to be used as a satisfactory candidate for oral administration) while avoiding ethical, time- and cost-consuming issues related to human and animal testing, numerous *in vitro* permeability screening models have been proposed (Caco-2 model, Artusson et al., 2001; PAMPA model, Kansy et al., 1998; PVPA model, Flaten et al., 2006b; Permeapad™, di Cagno et al., 2015; AMI-system, Berben et al., 2018b). Studies combining different *in vitro* permeability models with simulated intestinal fluids (SIFs) have been carried out by several research groups, and a special focus has been put on the impact that SIF-driven drug solubilisation and permeation have on drug absorption (Berben et al., 2018b; Bibi et al., 2015; Fischer et al., 2012; Naderkhani et al., 2015). Other studies have focused on the impact that pH variations in the intestine have on drug solubility and permeability, and on the interplay that occurs between the two (Sieger et al., 2017). The effect of the mucus layer on the permeability of drugs and formulations has been investigated both with respect to their diffusion through this layer alone (Fabiano et al., 2017; Shaw et al., 2005), as well as through *in vitro* barriers in the presence of this hydrophilic layer (Falavigna et al., 2018; Keemink and Bergström, 2018; Stappaerts et al., 2018).

In the present study, we aimed to combine all the investigations discussed above. In particular, we utilised our previously developed modification of the PVPA (Phospholipid Vesicle-Based Permeation Assay) barrier comprising mucus (namely, mucus-PVPA, Falavigna et al., 2018) as a model for the intestinal membrane to move one step further towards closer mimicking the *in vivo* environment by studying the impact that pH and fasted/fed state SIFs have on drug permeability, as well as their interplay with mucus. The

integrity of these liposome-based barriers was assessed in terms of permeability of a hydrophilic fluorescent marker and of the electrical resistance across the barriers at different pH and in the presence of different fasted and fed state SIFs. Subsequently, the solubility and permeability profiles of five model acidic/basic compounds were evaluated together with the investigation on the rheological behaviour of mucus at different pH conditions. Lastly, the permeability of a hydrophilic marker and a lipophilic BCS class II drug was examined in the presence of all commercially available versions of fasted and fed state SIFs.
Overall, the results collected in this study highlight the importance of assessing the impact that pH, mucus and fasted/fed SIFs have on drug permeability, and suggest the mucus-PVPA to be a promising tool for such purpose.

2. Materials and methods

2.1. Materials

Ammonium molybdate, calcein (CAL), chloroform, ethanol (96%, v/v), Fiske-Subbarow reducer, glacial acetic acid (≥99.8%), hydrochloric acid, ibuprofen (IBP), indomethacin (IND), maleic acid, methanol CHROMASOLV®, metoprolol (MTP), metronidazole (MTR), mucin from porcine stomach type III (bound sialic acid 0.5-1.5%, partially purified), naproxen (NPR), phosphorus standard solution, potassium phosphate monobasic, sodium chloride, sodium hydroxide, sodium phosphate dibasic dodecahydrate, sodium phosphate monobasic monohydrate and Triton X-100 were products of Sigma-Aldrich Chemie GmbH (Steinheim, Germany). E80 lipoid egg-phospholipids (80% phosphatidylcholine) were obtained from Lipoid GmbH (Ludwigshafen, Germany). Acetonitrile for HPLC (gradient grade) was a product of VWR chemicals (Fontenay-sous-Bois, France) and sulphuric acid

was purchased from May&Baker LTD (Dagenham, England). Hydrogen peroxide 30% and Titriplex® III were obtained from Merck KGaA (Darmstadt, Germany).
FaSSIF/FeSSIF/FaSSGF, FaSSIF-V2 and FeSSIF-V2 powders were purchased from biorelevant.com (Croydon, UK). All chemicals employed were of analytical grade.
For the preparation of the PVPA barriers, nucleopore track-etch membrane filters (0.4 and 0.8 µm pore size) were purchased from Whatman (part of GE Healthcare, Oslo, Norway) and the nitrocellulose membrane filters (0.65 µm DAWP) were obtained from Millipore (Billerica, Massachusetts, USA). Transwell filter inserts and plates (d = 6.5 mm) were products of Corning Inc. (Corning, New York, USA).

2.2. Drugs pH-dependent solubility studies

The solubility of different drugs (IBP, IND, MTP, MTR, NPR) was investigated at pH 5.5, 6.2 and 7.4 at room temperature (23-25 °C), following the method described by Berthelsen et al., (2014).
Briefly, phosphate buffer saline (PBS) was prepared in order to obtain three buffers with different final pH (5.5, 6.2 and 7.4). 15 mg of drug were dispersed in 15 mL of PBS in a 15 mL tube, and left to rotate on a Labinco test-tube rotor (Breda, The Netherlands) for a total of 24 hours. After 1, 4 and 24 hours, the tubes were centrifuged for 10 minutes at 4500 rpm on a Biofuge Stratos thermostated centrifuge (Heraeus Instruments GmbH, Hanau, Germany), where the temperature was kept between 23 and 25 °C to avoid sample heating and further drug solubilisation. 1 mL of the supernatant solution was further centrifuged for 10 minutes at 13000 rpm on a Biofuge pico centrifuge (Heraeus Instruments GmbH, Hanau, Germany), in order to provide an additional separation of the possible undissolved drug, thus making sure that the amount of drug quantified at the end of the experiment would only be the fraction

dissolved in the aqueous media. The supernatant was diluted and the amount of drug dissolved was quantified spectrophotometrically on SpectraMax 190 Microplate reader (Molecular Devices Corporation, California, USA). The 15 mL tubes were vortexed and put back on the rotor. For each drug and each pH, 2 samples were prepared and analysed to assess changes in solubility.

IBP, IND, MTP, MTR and NPR were quantified spectrophotometrically on SpectraMax 190 Microplate reader (Molecular Devices Corporation, California, USA) at wavelengths of 220, 254, 274, 320 and 270 nm, respectively.

2.3. PVPA barrier preparation

The PVPA barriers were prepared according to the method previously described by Naderkhani et al. (2014a). Briefly, egg-phospholipids (E80) liposomes were obtained by the film hydration technique and extruded to obtain liposomes with two different size populations by means of 0.8 and 0.4 µm pore size filters. The liposomes were then deposited by centrifugation on top of cellulose ester filters (0.65 µm pore size), followed by a freeze-thaw cycle to immobilize and fuse the liposomes to the filter support.

2.3.1. Mucus-PVPA barrier preparation

To assess the impact of the mucus layer on drug permeability, 50 µL of mucin dispersion were added on top of the PVPA barriers according to the method previously described by us (Falavigna et al., 2018). Briefly, mucin from porcine stomach type III was hydrated with PBS pH 7.4 in order to achieve a final concentration of 10 mg/mL or 40 mg/mL. The dispersion was directly pipetted on top of the PVPA barriers and was left to incubate for 5 minutes prior

to the addition of the drug/marker solution. When the impact of different pH on drug permeability was investigated, the mucin dispersion was adjusted to the investigated pH with the use of HCl or NaOH solutions before its addition on top of the barriers.

2.3.2. Mucus rheology

Rheology measurements of the mucus prepared with different concentrations of mucin (10 and 40 mg/mL) as well as at different pH (5.5, 6.2 and 7.4) were performed on a Discovery HR-2 hybrid rheometer (TA instruments, New Castle, USA) equipped with a Peltier plate environmental system, a cross hatched 40 mm parallel plate geometry, and a cross hatched lower plate. The sample was placed on the lower plate, the geometry was lowered to the measuring gap of 1000 µm, and the system was let equilibrate for 180 seconds at 25 °C (the same temperature at which the permeability experiments were performed). The viscosity of the different mucus simulating dispersions and the stress applied were measured using a logarithmic flow sweep with steady state sensing, where the shear rate was increased incrementally with 30 points per decade from 2 to 200 1/s. For each mucin concentration and each pH, three samples were prepared and measured.

2.4. Simulated intestinal fluids preparation

To study the effect of simulated intestinal fluids (SIFs) on the integrity of the PVPA barriers and on drug permeability, fasted (Fa-) and fed (Fe-) state SIFs were prepared according to the standardised protocol provided by the supplier (biorelevant.com). In this study, two versions (V1 and V2) of the simulated intestinal powders were used. Briefly, FaSSIF/FeSSIF/FaSSGF (producing FaSSIF-V1 or FeSSIF-V1), FaSSIF-V2 or FeSSIF-V2 powder was dissolved in

the corresponding fasted (FaB-V1 or V2) or fed (FeB-V1 or V2) buffer. The compositions of the different media are depicted in Table 1.

Table 1

2.5. *In vitro* permeability studies

The (mucus-)PVPA barriers were used to study the permeability of different drugs/marker at room temperature (23-25 °C) following the procedure previously described (Falavigna et al., 2018), in the presence and absence of a mucus layer at different pH conditions and using different dissolution media. When the experiment was carried out in the presence of mucus, 50 µL of mucin dispersion were added on top of the PVPA barriers and let to incubate for 5 minutes prior to the addition of the drug/marker in solution. After the drug/marker solution (100 µL) was added on top of the PVPA barriers/mucus layer, the inserts were placed in an acceptor compartment containing 600 µL of PBS pH 7.4, simulating the *in vivo* blood circulation. The inserts were moved to fresh acceptor compartments after 1, 2, 3, 3.5, 4, 4.5 and 5 hours in order to maintain sink conditions. After 5 hours, the samples were collected from the acceptor compartment prior to their quantification, and the electrical resistances of the barriers were measured to examine the integrity of the barriers.

IBP, MTP, MTR and NPR were spectrophotometrically quantified as described in 2.2. IND was quantified by HPLC-UV at a wavelength of 254 nm (retention time 3.05 min; injection volume: 20 µL) using a Waters X-select™ CSH ™ C18 (2.5 µm, 3.0 × 75 mm) XP column (guard cartridge: Waters X-select™ CSH ™ C18 3.5 µm, 3.0 × 20 mm) on a Waters e2795 Separation Module connected to a Waters 2489 UV/Visible Detector (Waters, Milford, Massachusetts, USA). The flow rate was adjusted to 0.5 mL/min and the mobile phase

consisted of acetonitrile and MilliQ water with 0.1% glacial acetic acid (60:40, v/v). CAL was quantified spectrofluorometrically at excitation and emission wavelengths of 485 and 520 nm, respectively, using a POLARstar Galaxy fluorometer (Fluostar, BMG Labtechnologies, Offenburg, Germany). Validation parameters, LOD and LOQ for the quantification of all compounds can be found in the Supplementary Material.

The apparent permeability coefficient (P_{app}) was calculated with the following equation, derived from Fick's law:

$$P_{app}\left(\frac{cm}{s}\right) = \frac{dQ}{dt} * \frac{1}{A * Cd}$$

where *dQ/dt* expresses the slope at the steady-state conditions (nmol/s), *A* is the surface area of the barriers (cm^2) and *Cd* represents the concentration of the drug/marker in the donor compartment (nmol/mL).

To ensure sink conditions, the drug/marker concentrations added in the donor compartment were selected in order to achieve a value below the solubility limit (< 10% of the donor concentration) in the acceptor compartment (Flaten et al., 2006a, b).

For each drug/marker in each condition, the permeability study was carried out at least in triplicate (6 PVPA barriers tested for each one of the three parallels).

2.5.1. The effect of pH on barrier integrity and drug permeability

To assess changes in P_{app} due to different pH conditions of the solution in the donor compartment, several drugs/marker (i.e. CAL, IBP, IND, MTP, MTR and NPR) were dissolved in PBS pH 5.5, 6.2 or 7.4. In the case of the permeability experiment in the presence of mucus, mucin 10 mg/mL was prepared according to the pH of the drug/marker solution.

In particular, as an increase in the permeability of the fluorescent marker CAL would indicate possible disruption of the barriers (Flaten et al., 2006b; Naderkhani et al., 2015), its permeability was quantified to investigate the impact of changes in pH on the integrity of the barriers.

2.5.2. The effect of simulated intestinal media on barrier integrity and drug permeability

To investigate the impact of the SIFs on the integrity of the barrier and on the permeability of drugs, CAL and IBP were dissolved in FaB, FeB, FeSSIF or FaSSIF (V1 and V2), and their P_{app} was evaluated in the presence and absence of mucus (mucin 10 mg/mL). The mucus layer was prepared in accordance with the pH of the media.

As the P_{app} of CAL dissolved in the fasted media was exceeding the standard range (Flaten et al., 2006b), the influence of mucin 40 mg/mL on the permeability of CAL was investigated to examine its potential to act as a further protective layer for the PVPA barriers.

Moreover, the same conditions studied by Fischer et al. (2012) were investigated in this study. Briefly, the PVPA barriers were incubated for 1 hour in FaB (V1 or V2) prior to the addition of the CAL solution in the fasted buffers/media (FaB, FaSSIF V1 and V2), and the permeability of CAL was quantified for a total of 4 hours following the procedure described in section 2.5.

2.6. Phospholipid quantification

The amount of phospholipids lost from the PVPA barriers in the presence of fasted and fed state buffers/media was quantified using the modified phosphorus assay (Bartlett, 1959),

following the method previously described by Naderkhani and colleagues (Naderkhani et al., 2015). Briefly, the PVPA barriers were placed in an acceptor compartment containing 600 µL of PBS pH 7.4 and the donor compartment was loaded with FaB, FeB, FaSSIF or FeSSIF (V1 and V2) (100 µL). The barriers were incubated for 5 hours. The incubations in PBS pH 7.4 and 0.5 % Triton X-100 were used as negative and positive control, respectively. Samples (50 µL) were withdrawn from the donor compartment after 5 hours, diluted with 50 µL of distilled water and treated following the phosphorus assay. Blanks (PBS pH 7.4, 0.5 % Triton X-100, FaB, FeB, FaSSIF, FeSSIF, V1 or V2) were treated in the same manner. Three PVPA barriers for each condition were tested.

2.7. Statistical analysis

The statistical evaluation of all results was carried out using GraphPad Prism 7.0 software. When significant difference between two sets of data was to be highlighted, student-t test was employed ($p < 0.05$). One-way ANOVA was used to compare three or more sets of data and the Bonferroni *post hoc* test was employed to detect significant differences ($p < 0.05$).

3. Results and discussion

3.1. The pH environment of the intestinal tract

Drug solubility and permeability in the intestinal tract are regarded as the two major factors affecting oral drug absorption, especially with regards to poorly soluble compounds. As the pH environment of the intestine varies widely through its length (5.6-7.8, Bergström et al., 2014), it is of key importance to investigate its effect on solubility and permeability. In order

to infer if a pH-dependent solubility/permeability trend could be observed for drugs with different physicochemical characteristics, we studied the impact that a shift in pH could have on the solubility and permeability of five different drugs. Moreover, as drug permeability was assessed both in the presence and absence of a mucus layer at pH 5.5, 6.2 and 7.4, the investigation on the integrity of the PVPA barriers and the rheological characterisation of mucus in such pH conditions were carried out.

3.1.1. Barrier integrity and mucus characterization

To guarantee the optimal functionality of the (mucus-)PVPA barriers, their integrity was investigated at pH 5.5, 6.2 and 7.4. CAL was chosen as a marker to detect changes in barrier integrity at the selected pH conditions both in the presence and absence of the mucus layer. Fig. 3a (shaded area) shows that no significant increase in CAL permeability was found compared to the reference value ($0.06 * 10^{-6}$ cm/s; Flaten et al., 2006b; Flaten et al., 2008), suggesting that the investigated conditions did not cause any barrier impairment. The electrical resistance across the barriers was measured after 5 hours, and the results (data not shown) also indicated intact barriers (electrical resistance > 290 Ohm*cm^2, Naderkhani et al., 2015).

Since drug permeability in the intestinal environment could be affected by the rheology of the mucus layer, and as it has been demonstrated that mucus can undergo a conformational change induced by a shift in pH (Lieleg et al., 2010), rheology measurements of the mucus placed on top of the PVPA barriers were carried out at different pH conditions and mucin concentrations.

As it can be observed in Fig.1, the general Newtonian behaviour of the mucus at pH 6.2 (10 mg/mL) and 7.4 (10 and 40 mg/mL) confirmed previous findings regarding mucus rheology (Falavigna et al., 2018; Mackie et al., 2017). However, when decreasing the pH of the mucin hydration media to 5.5, a non-Newtonian (shear-thinning) behaviour was observed (Fig. 1), correlating with what other research groups have found in the *in vivo* mucus layer (Boegh et al., 2014; Lai et al., 2009). These findings show how the rheology of mucus could be affected by the change in environmental pH. In fact, Cao et al., (1999) have suggested that a sol-gel transition could result from a pH-induced conformational change when decreasing the pH from 6-7 to a more acidic one. Lieleg et al., (2010) have also proposed that, at lower pH, the mucus layer tends to generate a stronger barrier toward particle mobility compared to a neutral pH environment. With regards to mucus viscosity, Fig. 1 shows that a decrease in pH or an increase in mucin concentration, causes an increase in apparent viscosity, as previously observed in other studies (Cao et al., 1999; Park et al., 2007).

The results obtained in this study prove that mucus can undergo relevant rheology changes, which should be carefully taken into account when assessing the behaviour of a drug in such environment. These considerations are especially relevant when investigating the diffusion of drugs and formulations through the mucus layer and their subsequent permeation through the intestinal mucosa.

Fig. 1

3.1.2. Drugs pH-solubility profiles

The pH-dependent permeability profiles of five model drugs were evaluated using the mucus-PVPA model. The selection of the drugs was carried out to cover both acidic (IBP, IND, MTR and NPR) and basic (MTP) compounds as well as compounds with different degree of

lipophilicity (Table 2). Since the pH-dependent solubility of a drug is important when investigating its ability to permeate the GI barrier, solubility studies of the five model drugs were performed at pH conditions simulating different parts of the intestinal tract.

Table 2

As it can be observed from Fig. 2 and Table 2, the equilibrium solubility of the investigated drugs at the different pH conditions was dependent on their acidity constant (pKa), the pH of the medium in which the drugs were solubilized and their intrinsic hydrophilicity/lipophilicity. In particular, for IBP, IND and NPR (acidic and lipophilic drugs with pKa ≈ 4 and LogP > 3), the solubility significantly increased ($p < 0.05$) from pH 5.5 to 7.4, as their degree of ionization increases at pH higher than their isoelectric point. In the case of the basic drug MTP (pKa 9.56, LogP 1.88), a non-significant decrease in solubility was found when increasing the pH to 7.4. This finding is most likely related to the fact that the pH conditions at which the experiments have been performed were far below the isoelectric point of MTP, thus not significantly differentiating the solubilities at pH 5.5, 6.2 and 7.4. For MTR (pKa 2.62, LogP -0.02), a significant increase was only observed when comparing the solubility at pH 5.5 with the other two pH conditions. Again, this is most likely due to the fact that solubility changes are only observable when comparing pH closer to the isoelectric point. Moreover, the hydrophilicity/lipophilicity of the examined drugs highly influenced their solubility. The more hydrophilic compounds such as MTP and MTR were found to be more soluble compared to more lipophilic IBP, IND and NPR, as expected.

Fig. 2

These findings are in accordance with previous pH-dependent investigations carried out both *in vitro* and *in silico* (Bergström et al., 2004; Shoghi et al., 2013; Völgyi et al., 2010; Varma et al., 2012).

Nonetheless, it has to be noted that the solubility profiles are substance-specific and that not only the pH, but also the ionic strength and the buffer capacity of the environment simulating the intestinal media should be carefully considered (Bergström et al., 2014; Hamed et al., 2016; Madsen et al., 2018).

3.1.3. Drugs pH-permeability profiles

The P_{app} of the same five model drugs was examined in the presence and absence of mucus layer at different pH conditions (5.5, 6.2 and 7.4) to investigate their possible pH-dependent permeability.

In general, the permeability of the investigated compounds in the absence of the mucus layer was found to be highly affected by their degree of ionization (Fig. 3), in accordance with the pH partition hypothesis (Shore et al., 1957). It has previously been shown that an increase in the fraction of drug in its unionized form directly increases the permeability of the drug (Flaten et al., 2008; Shore et al., 1957). In particular, in our study it was found that, for the BCS class II acidic drugs IBP and NPR, the permeability significantly decreased ($p < 0.05$) with increasing pH of the donor compartment, as the ionized form became the predominant one. Correspondingly, the permeability of the BCS class I basic drug MTP exhibited an increase in P_{app} when the pH was increasing from 5.5 to 7.4. For the BCS class II IND, the decrease in P_{app} with increasing pH was less visible, probably due to the highly lipophilic nature of the compound, which can cause a retention of the drug into the barriers and thereby causing a low recovery at the end of the experiment (Naderkhani et al., 2015). With regards to

BCS class I MTR, no change in permeability was found at different pH conditions. This was most likely due to the fact that the pH conditions of the experiments were significantly above the isoelectric point for this acidic compound, in accordance with the solubility results discussed in section 3.1.2. Furthermore, it has to be noted that more lipophilic compounds such as IBP and NPR (LogP > 3) are able to permeate the lipophilic PVPA barriers to a higher degree compared to more hydrophilic ones such as MTP and MTR due to their intrinsic nature.

The (mucus)-PVPA has previously shown to correlate well with *in vivo* data on the fraction absorbed in humans (Flaten et al., 2006b; Naderkhani et al., 2014b; Falavigna et al., 2018). Furthermore, satisfactory correlations were found between the results obtained in this study at the different pH conditions (7.4, 6.2 and 5.5) and data where drug permeability was assessed at comparable pH (6.83) using a mucus-comprising Caco-2 cell model (Fig.2S and 3S, Supplementary Material). Especially in the case of the (mucus)-PVPA data at pH 7.4, which was the pH closest to the one used in the Caco-2 cell experiments, R^2 of 0.96 and 0.97 were identified in the presence and absence of mucus respectively.

Fig. 3

As the intestinal walls are lined with a mucus layer that differs in pH according to the specific location (Lieleg et al., 2010), the permeability of the same compounds was tested in the presence of mucin 10 mg/mL to assess the impact of this additional layer at different pH conditions. With regards to mucus-drug interaction, it has to be noted that there are multiple mechanisms which could take place when different drugs are in contact with this layer. In particular, Olmsted and colleagues (Olmsted et al., 2001) suggested interaction filtering (depending on specific binding interactions, electrostatic and hydrophobic forces and hydrogen bonds) and size filtering as the two main driving forces for the diffusion of drugs

through the hydrophilic mucus layer. This emphasizes the fact that more lipophilic compounds might decrease their rate of diffusion through mucus to a higher extent compared to hydrophilic ones, and that their ionization might further be the driving force according to the pH environment (Khanvilkar et al., 2001; Shaw et al., 2005).

When the hydrophilic mucus layer was added on top of the PVPA barriers, the permeability of the different drugs was generally decreased compared to its absence, and the pH effect was also less evident (Fig. 3). As the isoelectric point of mucin is estimated to be between 2 and 3 (Lee et al., 2005), its ionization would increase with the increase in pH. When the same occurs for ionisable drugs, this could cause an electrostatic repulsion or interaction (according to the nature of the drug) that would translate into a decrease in the P_{app} of the drug (Shaw et al., 2005).

Moreover, the lipophilicity of the drug might also affect its degree of interaction with the mucus layer. In particular, the permeability of the more lipophilic compounds IBP and NPR significantly decreased ($p < 0.05$) in the presence of the mucus layer compared to its absence in all tested pH conditions.

Additionally, changes in the rheological characteristics of mucus, usually occurring with a shift in environmental pH, could affect the diffusion/permeability behaviour of drugs at different pH conditions. In fact, as it can be observed in Fig. 3 for IBP and NPR, the higher viscosity of the mucus at pH 5.5 (Fig. 1) could be a contributing factor to the greater decrease in permeability compared to the results at pH 6.2 and 7.4. The findings obtained in this study highlight how the inclusion of the mucus layer is of key importance when investigating pH-dependent permeability, and emphasize the mucus-PVPA model as a suitable tool to study drug permeation in the intestinal environment.

The permeability-solubility interplay was studied by plotting the permeability and the solubility of the different drugs previously investigated against the pH in the absence of mucus (Fig. 4). A similar trend would be visible by plotting the results in the presence of the mucus layer. As it can be observed, for acidic drugs with pKa around 4 (IBP, NPR and IND; Fig. 4A, B, C) the permeability was higher at more acidic pH, whereas their solubility showed the opposite pH-dependent trend. On the other hand, a pH-driven variation in solubility and permeability was not noticeable for MTR (Fig. 4D), as expected from its physicochemical characteristics (Table 2). For the basic drug MTP (Fig. 4E), the tendency of higher permeability at decreasing degree of ionization was observed, but a significant decrease in solubility was not visible.

The trends observed can be explained by the pH partition hypothesis, which highlights the fact that ionisable drugs tend to permeate lipidic membranes when in their undissociated form (Shore et al., 1957), whereas their solubility is higher when the dissociated form is the predominant one. Moreover, these findings are in agreement with previous investigations on the pH-dependent permeability-solubility interplay for ionisable compounds (Sieger et al., 2017).

Since previous findings have emphasized that the solubility-permeability trade-off should be carefully considered when aiming to design optimal formulations (Dahan and Miller 2012; Porat and Dahan 2018), it is essential to combine permeability and solubility *in vitro* tools to elucidate this interplay. The PVPA model used in this study, together with pH-dependent solubility experiments, proved the relevance of this kind of investigation and showed to be appropriate for such purpose.

Fig. 4

3.2. The intestinal media environment

Together with the variations in environmental pH, the intestine is also characterized by intraluminal fluids that can vary in composition according to the fasted or fed state (Clarysse et al., 2009).

In particular, bile salts and lecithin have shown to form colloidal structures which can provide the entrapment of drug molecules and their subsequent increased solubilisation, especially with regards to lipophilic drugs (Augustijns et al., 2014; Jantratid et al., 2008; Dahan and Miller, 2012). The fraction of the drug solubilised by these structures is not readily able to permeate the intestinal walls (Miller et al., 2011) and, for this reason, it is important to assess the impact that intestinal fluids have on drug permeation. The commercially available FaSSIF and FeSSIF have previously been proved to mimic the composition of the human intestinal fluids (Jantratid et al., 2008) and have been extensively used in the past decade in numerous solubility and permeability studies using artificial cell-free permeability models (Berben et al., 2018b; Bibi et al., 2015; Fischer et al., 2012; Naderkhani et al., 2015). Therefore, in this study we evaluated the impact that these SIFs have on the PVPA barriers, as well as on the permeability of different compounds.

3.2.1. PVPA barriers in the presence of simulated intestinal media

The PVPA barriers used in this study have previously been shown to be stable in the presence of FaSSIF V1 by another research group (Fischer et al., 2012). However, as the components in the different SIFs could potentially interact with the PVPA lipids and affect the integrity of the barriers, we wanted to investigate this further. For the first time, in this study the PVPA and mucus-PVPA barriers were evaluated in terms of their compatibility with both the fed and

fasted state SIFs (namely, FeSSIF and FaSSIF, V1 and 2, composition found in Table 1). To the best of our knowledge, this is the first attempt in studying the impact that all the commercially available media versions (version 1, V1; version 2, V2) have on the functionality of the PVPA barriers; we believe that this investigation is crucial in order to design the best intestinal-resembling *in vitro* permeability model.

The permeability of the fluorescent marker CAL was used to evaluate if the addition of the fed or fasted state SIFs would induce changes in the integrity of the PVPA barriers.

As previously mentioned, an increase in the reference calcein P_{app} value ($0.06 * 10^{-6}$ cm/s) and decrease in barrier electrical resistance (< 290 Ohm*cm^2) would suggest a potential change in barrier integrity (Flaten et al., 2006b; Flaten et al., 2008; Naderkhani et al., 2015).

As it can be observed in Fig. 5, with the fed state buffers/media (Fig. 5A) the permeability of CAL did not increase compared to the control (PBS pH 5.5, Fig. 5A shaded area), suggesting that their presence did not influence the functionality of the barriers, both in the presence and absence of the mucus layer. On the other hand, a general increase in P_{app} and decrease in electrical resistance was observed when experiments with CAL dissolved in the fasted state buffer/media were performed (Fig. 5B). However, permeability of CAL was lower in the presence of buffers compared to the fasted state media, suggesting that the components found in the media (namely sodium taurocholate, lecithin, glycerol monooleate and sodium oleate; Table 1) could be causing changes in the integrity of the barriers.

The presence of mucin 10 mg/mL seemed to shield the barriers from the effect of FaSSIF V1, which was the medium causing the most significant change in CAL permeability. Therefore, to test if mucus with a higher mucin concentration would provide additional protection of the barriers, mucin 40 mg/mL was also tested. As it can be seen from Fig. 5B, in general this setup led to a decrease in CAL P_{app}, especially in the case of the fasted state media, suggesting a higher degree of protection from the more concentrated mucus layer. However, CAL

permeability was still significantly higher compared to the control and the electrical resistance measured in this condition was still below the optimal range (< 290 Ohm*cm^2; Naderkhani et al., 2015).

As mentioned above, Fischer and colleagues (Fischer et al., 2012) concluded that the integrity of the barriers appeared to be maintained in the presence of the fasted state medium (V1) since the results obtained using FaB and FaSSIF (V1) were not statistically different. In this study, PBS pH 7.4 was not included as control. The permeability experiments performed by Fisher and colleagues were carried out in a different manner compared to the present study. In particular, the barriers were hydrated for 1 hour in FaB V1 and the following permeability assay was 4 hours long. For this reason, we decided to investigate the permeability of CAL in these conditions. As it can be observed in Fig. 5B, a significant decrease in calcein P_{app} was found with the hydrated-barriers setup, especially with the V1 fasted state medium. These findings, together with the differences in surface area and donor volume, as well as lab-to-lab variations, could be the reasons for the differences between the results obtained in the current study and the ones from Fischer and colleagues (Fischer et al., 2012).

Moreover, in previous studies performed in our research group (Naderkhani et al., 2015) a modification of the original PVPA model (namely, $PVPA_{biomimetic}$) was used to assess the impact of the fasted and fed state SIFs (V2). In accordance with our findings (Fig. 5), a higher CAL permeability and lower electrical resistance was observed in the presence of fasted state medium compared to the fed one (Naderkhani et al., 2015). However, with the $PVPA_{biomimetic}$ the fasted medium (V2) was found to be much less aggressive to the barriers and thus more compatible with the model compared to the original PVPA (Naderkhani et al., 2015). The $PVPA_{biomimetic}$ barriers have also shown to be more robust against the presence of co-solvents and tensides compared to the original PVPA (Naderkhani et al., 2014b), and are thus a good

alternative when permeability studies with conditions that might affect the original PVPA barriers have to be performed.

Fig. 5

However, as the P_{app} of drugs/compounds can be differently affected according to their physicochemical characteristics, we wanted to investigate how the permeability of one more lipophilic compound would be affected in the presence of the SIFs. Therefore, the permeability of the BCS class II drug IBP was evaluated in the presence of fed and fasted state SIFs with and without the presence of the mucus layer, to see if the variation in the permeability of IBP would follow the same trend as the one of the hydrophilic marker CAL. As it can be seen in Fig. 5D, the permeability of IBP dissolved in the fasted state media in the absence of mucus significantly increased ($p < 0.05$) only in the case of FaSSIF V2, when compared to the one where the drug was dissolved in PBS pH 6.2 (Fig. 5D, shaded area). In the presence of the mucus layer, the corresponding buffer (FaB V2) also caused a significant increase in P_{app}. The electrical resistance across the PVPA barriers followed the trend seen in Fig. 5B, suggesting potential barrier impairment in the presence of the fasted state media. However, the permeability of this lipophilic compound was not affected by the presence of this media to the same extent as CAL, suggesting that the changes in the PVPA structure may be related to an increase in aqueous pores through the barriers and not to variations in their lipidic part. In fact, events that affect the structure of the PVPA barriers can cause an increase in aqueous pathways, resulting in a higher permeability especially for hydrophilic compounds (Flaten et al., 2006b).

Fig. 5C shows the apparent permeability of IBP dissolved in the fed state (SIFs). In general, minor changes were found when comparing the P_{app} of the drug dissolved in PBS pH 5.5 (control, shaded area) with the one in the fed state buffers/media in the presence and absence

of the mucus layer. These findings could be related to the different solubilisation that the drug can exhibit in these different environments, which again can translate into a change in permeability (Dahan and Miller 2012; Porat and Dahan 2018). Moreover, in all tested fed conditions, the P_{app} of IBP was found to be lower in the presence of mucus. On the contrary, as it can be seen in Fig. 5A, the permeability of the hydrophilic marker CAL did not significantly change between the presence and absence of mucus. In fact, the presence of the mucus layer can particularly hinder the diffusion of lipophilic drugs because of its hydrophilic nature and of the possible interaction of the drugs with its hydrophobic regions (Khanvilkar et al., 2001). These results were able to prove this concept, and stressed the necessity of a permeability *in vitro* model comprising mucus to properly assess its impact on oral drug absorption.

As previously mentioned, lecithin and bile salts have shown to entrap drug molecules in vesicular structures, thus increasing drug solubilization and lowering the amount of free drug able to permeate through the intestinal walls (Augustijns et al., 2014; Miller et al., 2011). This effect should be particularly evident with the fed state media, where the concentration of the above-mentioned components is higher compared to the fasted state one. As a proof of this concept, Fig. 5C shows a significant decrease ($p < 0.05$) in permeability when comparing the P_{app} of IBP dissolved in FeB V2 with the one in FeSSIF V2, both in the presence and absence of mucus. In fact, the presence of sodium taurocholate, lecithin and other lipolysis products in FeSSIF V2 can provide the formation of micelles, otherwise not present in the fed state buffer (FeB V2), thus influencing drug solubilisation and permeability. A similar trend was found with FeB V1 and FeSSIF V1, but not to the same extent. This could be due to the different composition of the two media (Table 1), stressing the significant impact on drug permeation of the presence of FeSSIF V2, which has a higher bile salt-lecithin ratio and additional lipolysis products (sodium oleate and glycerol monooleate). Regarding this matter, it has been

previously shown that the complex composition of the fed intestinal fluids could contribute to larger colloidal vesicles (Riethorst et al., 2016) and therefore affect drug absorption to a higher extent. Moreover, according to the bile salt-lecithin ratio, the vesicles could change in dimension and tend either to a bilayered structure or to the one of mixed micelles, as previously discussed (Riethorst et al., 2018). The same trend was not observed for CAL (Fig. 5A), emphasising that this study was able to highlight the fact that the FeSSIF composition did not affect the permeability of hydrophilic compounds in the same manner as lipophilic ones.

In the presence of mucus, a significant decrease in IBP permeability ($p < 0.05$) was only found between FeB and FeSSIF V2 and not between FeB and FeSSIF V1 (Fig. 5C). This could again be traced back to the different composition of the two media, as well as to the potential interaction of the drug with the mucus. Moreover, the reduction in drug diffusion through native mucus has previously been found to be related to sodium taurocholate, competing with mucins in binding the drug diffusing through this layer (Legen and Kristl 2001).

All these considerations underline the impact that the different SIFs have on the permeation of different compounds through the PVPA barriers, but also on the diffusion of drugs through the mucus layer. These findings are especially relevant as the need of predictive *in vitro* models simulating the GI tract is further increasing (Berben et al., 2018a; Billat et al., 2017; Riethorst et al., 2018).

3.2.1.1. Loss of lipids from the PVPA barriers in the presence of simulated intestinal media

To further investigate the mechanism behind the possible change in barrier integrity suggested by the increased CAL permeability discussed in section 3.2.1, the potential loss of lipids from the PVPA barriers in the donor compartment was investigated in the presence of the different SIFs.

Fig. 6 shows how the loss of phospholipids in all tested conditions was significantly lower ($p < 0.05$) than the one caused by the presence of Triton X-100 0.5% (positive control), which is certainly causing barrier disintegration as proved by other authors (Fisher et al., 2011; Naderkhani et al., 2015). However, an increase in amount of phospholipids released from the barrier was observed with some of the SIFs compared to the presence of PBS pH 7.4 (negative control). In particular, a significant increase in phospholipid loss compared to the negative control was observed in the presence of FaB V1, FaSSIF V1 and FaSSIF V2. Moreover, a significant difference in lipid loss was found between the buffer and the medium for the fasted state V2 and the fed state V1.

These trends could explain part of the permeability results shown in Fig. 5B and add more information regarding the effects of the SIFs on the tightness of the PVPA barriers. In particular, a higher loss of lipids is suggested in the presence of the fasted state media, compared to the fed ones, in accordance with the results discussed in section 3.2.1. Moreover, the results in Fig. 6 are in the same range as the ones previously observed by Fischer and colleagues (Fischer et al., 2012). However, they did not compare the loss of phospholipids caused by the fasted state media with the one in the presence of PBS pH 7.4, therefore a negative control as the one discussed in our study was not accessible.

Naderkhani et al. (2015) observed that a lower lipid loss was found with the $PVPA_{biomimetic}$ barriers both in the presence of fasted and fed state media and of Triton X-100 0.5% compared to the original PVPA, highlighting the difference in barrier integrity and further supporting the permeability results discussed in section 3.2.1.

In the present study, the loss of phospholipids caused by the fed and fasted state buffer/media in the presence of the mucus layer was also investigated. However, the collection process of the samples from the donor compartment led to variations in the amount of mucus present in each sample. As mucus was prepared in phosphorus-containing buffer (PBS), the amount of phosphorus quantified in each sample varied according to the amount of mucus withdrawn from the donor compartment, thus leading to compromised sensitivity of the assay and the results could therefore not be trusted in the presence of this layer.

Fig. 6

The different results observed using the FaSSIF and FeSSIF media could be ascribed to their different composition (Table 1), which is not only related to the different amounts of bile salts, lecithin and other lipolysis products, but also to their different buffer composition. In fact, as it can be observed in Fig. 5 and 6, in some cases the buffers themselves seemed to potentially affect the barrier integrity.

Moreover, since it has been reported that the concentration of bile salts and lecithin in fasted state human intestinal fluid is much lower compared to the fed one (Clarysse et al., 2009) and since the SIFs have shown to mimic the human intestinal fluids (Jantratid et al., 2008), the resulting vesicular structures would be different according to FaSSIF or FeSSIF media, thus possibly affecting the PVPA barrier structure in a different manner.

Overall, the results obtained suggest the PVPA barriers to be especially stable in the presence of the fed state media, whereas the ones found with the fasted state media suggest a certain potential of barrier impairment, and precautions should be taken when interpreting results obtained in presence this media. However, as the $PVPA_{biomimetic}$ barriers have shown to be

more robust compared to the original ones (Naderkhani et al., 2014b), they could be the best model to use when fasted state SIFs have to be employed to assess drug permeability. Moreover, the findings discussed in section 3.2.1 highlight the fact that different media can result in a different impact on the PVPA barrier integrity as well as on the permeability of the model compounds. This emphasises the relevance of the investigation on both media and both versions carried out in this study.

4. Conclusions

In this study, the impact of regional and nutritional intestinal differences has been successfully investigated using the mucus-PVPA *in vitro* model. The pH-dependent drug permeability and solubility profiles showed trends in agreement with the pH partition hypothesis. An increase in mucus viscosity at lower pH conditions was also observed. Moreover, the impact of bile salts and phospholipids on drug permeation was evident, and the different SIFs showed to influence the permeability to various extents according to the hydrophilicity/lipophilicity of the drugs. Further, the presence of mucus particularly affected the permeability of the more lipophilic compounds. The results obtained in this work thus suggest the suitability of the mucus-PVPA model for investigations on the impact that pH and SIFs, as well as their interplay with mucus, have on intestinal drug absorption.

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Conflict of interest

No conflicts of interest are declared by the authors.

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Figure and table captions

Fig. 1: Viscosity of mucus at different pH conditions (5.5, 6.2 and 7.4) and mucin concentrations (10 mg/mL and 40 mg/mL).

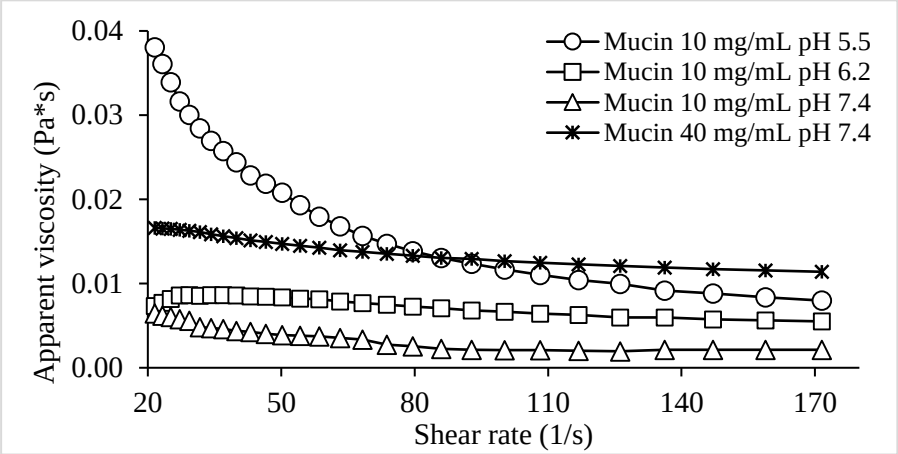

Fig. 2: Solubility of metronidazole (MTR), metoprolol (MTP), naproxen (NPR), ibuprofen (IBP) and indomethacin (IND) at pH 5.5, 6.2 and 7.4. The results are indicated as mean ± SD (n = 6). Statistical significance ($p < 0.05$) was investigated with one-way ANOVA using the Bonferroni *post hoc* test.

*statistically significant difference in solubility between pH 5.5 and 6.2/7.4.

**statistically significant difference in solubility between pH 6.2 and 5.5/7.4.

***statistically significant difference in solubility between pH 7.4 and 5.5/6.2.

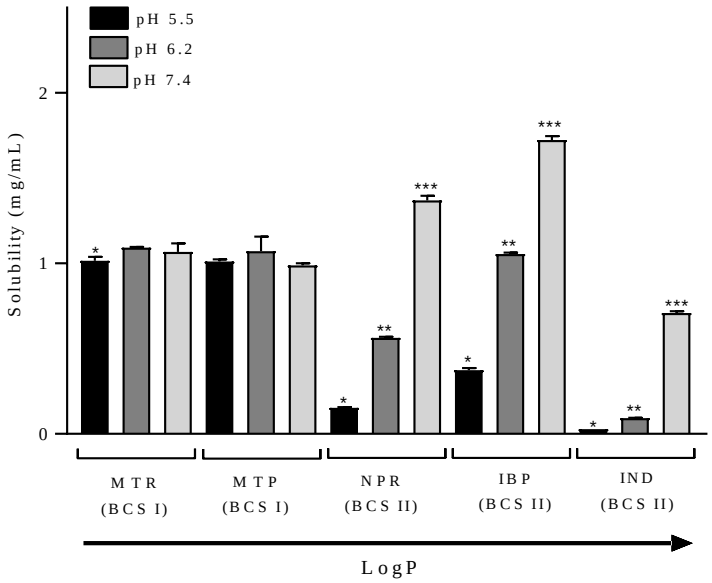

Fig. 3: Apparent permeability (P_{app}) of metronidazole (MTR), metoprolol (MTP), naproxen (NPR), ibuprofen (IBP) and indomethacin (IND) in the presence and absence of mucus at pH 5.5, 6.2 and 7.4. P_{app} of calcein (CAL) (shaded area) was quantified to investigate the integrity of the PVPA barriers at the chosen pH conditions. The results are indicated as mean ± SD (n = 18). Statistical significance ($p < 0.05$) was investigated with one-way ANOVA using the Bonferroni *post hoc* test.

* statistically significant difference in P_{app} between different pH conditions in the absence of mucus.

** statistically significant difference in P_{app} between different pH conditions in the presence of mucus.

° statistically significant difference in P_{app} between the presence and absence mucus.

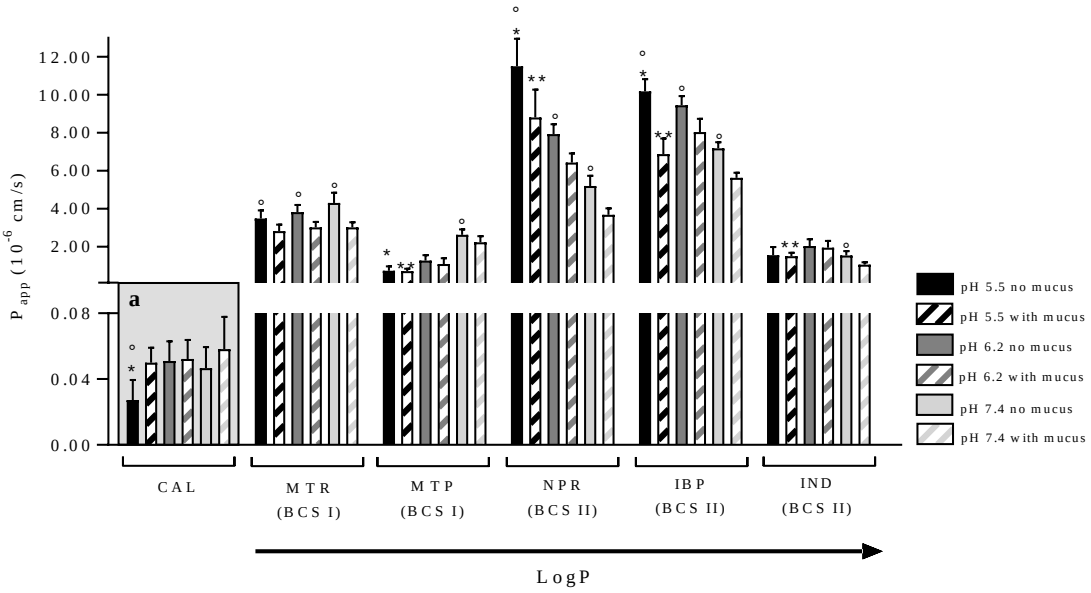

Fig. 4: pH-dependent permeability-solubility interplay in the absence of mucus, depicted as apparent permeability (P_{app}, black dotted line) and solubility (grey dotted line) plots of ibuprofen (4A, IBP), naproxen (4B, NPR), indomethacin (4C, IND), metronidazole (4D, MTR) and metoprolol (4E, MTP).

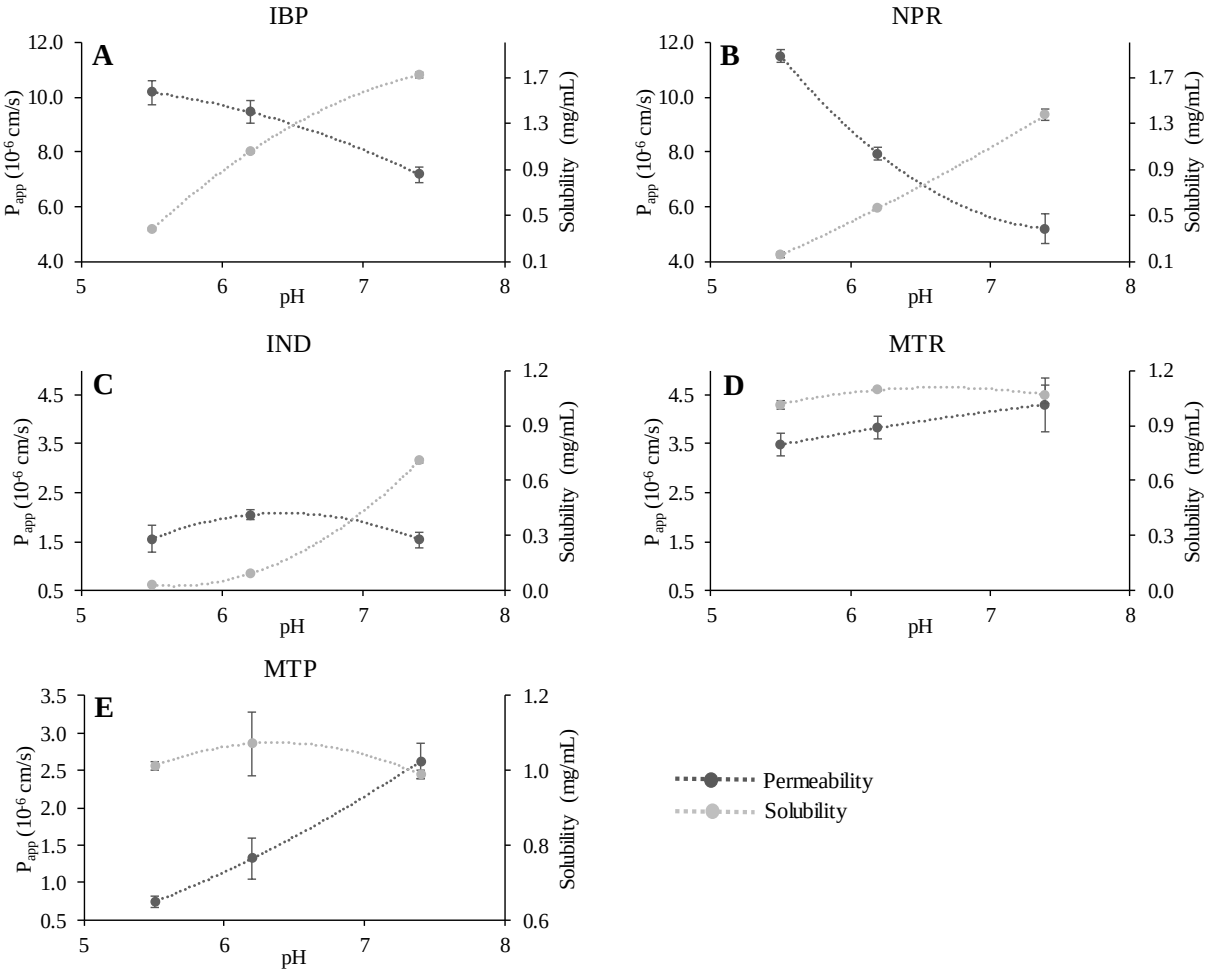

Fig. 5: Apparent permeability (P_{app}) of calcein (CAL; 5A, B) and ibuprofen (IBP; 5C, D), and electrical resistance of the PVPA barriers in the presence of fed (A, C) or fasted (B, D) state media (FeSSIF and FaSSIF, respectively) and corresponding buffers (FeB and FaB, respectively) for both versions 1 and 2 (V1, V2) in the presence and absence of mucus (10 and 40 mg/mL) and with hydrated barriers. P_{app} and electrical resistance in the presence of PBS (pH 5.5 or 6.2) are used as controls (shaded area). The results are indicated as mean ± SD (n = 18). Statistical significance ($p < 0.05$) was investigated with one-way ANOVA using the Bonferroni *post hoc* test.

$^{+}$ statistically significant difference in P_{app} between the absence of mucus and the other 3 conditions (mucus 10 mg/mL, mucus 40 mg/mL and hydrated barriers).

* statistically significant difference in P_{app} between PBS and all other SIFs without mucus.

** statistically significant difference in P_{app} between PBS and all other SIFs with mucus.

° statistically significant difference in P_{app} between the presence and absence of mucus.

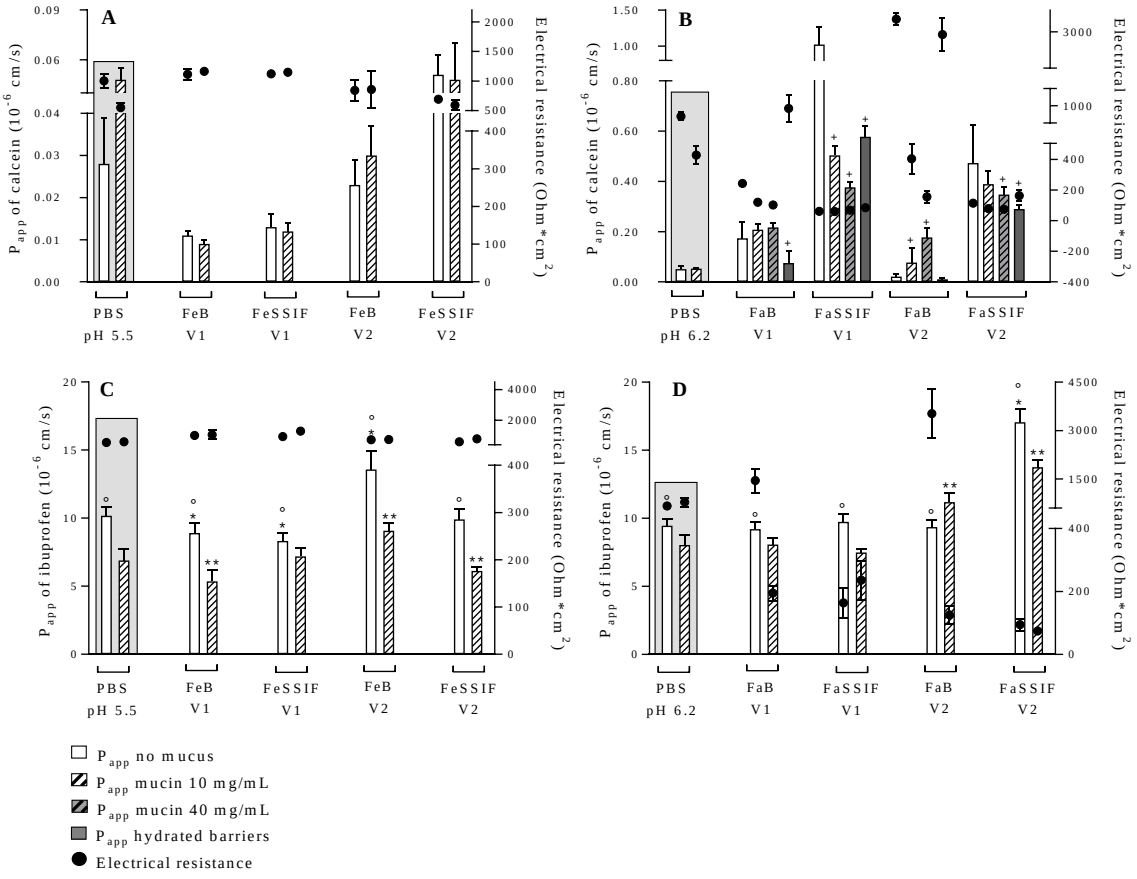

Fig. 6: Amount of phospholipids released to the donor compartment of the PVPA barriers after 5 hours of incubation with PBS pH 7.4, fasted (Fa-) and fed (Fe-) state buffers (FaB and FeB) and media (FaSSIF and FeSSIF) (both version V1 and V2) and Triton X-100 0.5%. The results are indicated as mean ± SD (n = 6). Statistical significance ($p < 0.05$) was investigated with one-way ANOVA using the Bonferroni *post hoc* test.

* statistically significant difference in phospholipids loss compared to PBS pH 7.4.

** statistically significant difference in phospholipids loss between the buffer and the media fluids.

° statistically significant difference in phospholipids loss between the presence of Triton 0.5% and all other conditions.

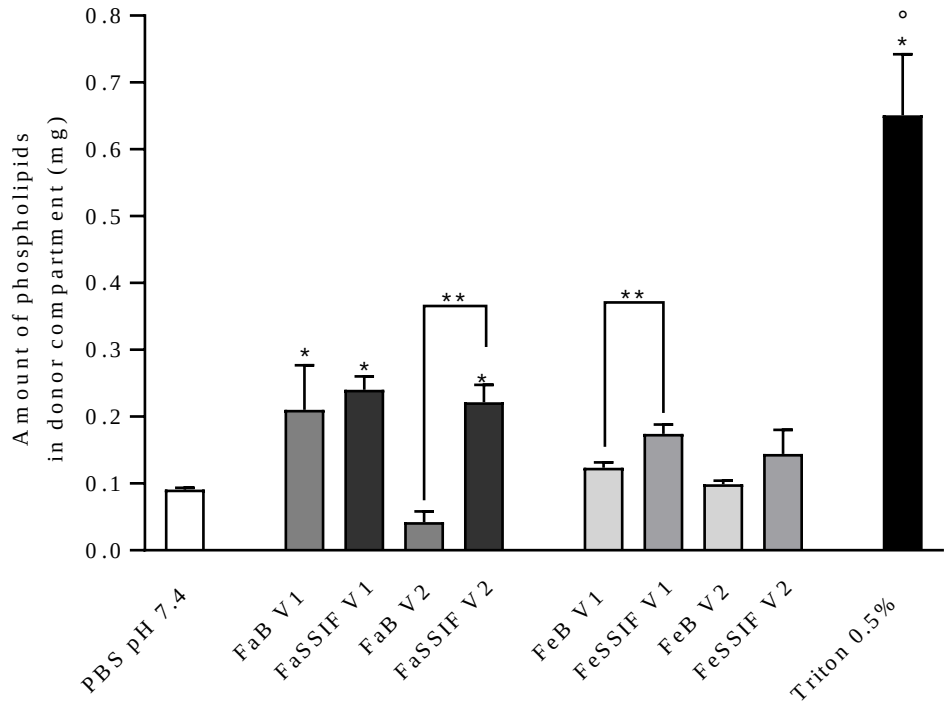

Table 1: Composition of the fasted (Fa-) and fed (Fe-) state simulated intestinal blank buffers (FaB, FeB) and media (FaSSIF, FeSSIF) for both version 1 and version 2 (V1, V2), as described by the provider (biorelevant.com).

	FaB-V1	FaSSIF-V1	FaB-V2	FaSSIF-V2	FeB-V1	FeSSIF-V1	FeB-V2	FeSSIF-V2
Sodium taurocholate (mM)	-	3.00	-	3.00	-	15.00	-	10.00
Lecithin (mM)	-	0.75	-	0.20	-	3.75	-	2.00
Glycerol monooleate (mM)	-	-	-	-	-	-	-	5.00
Sodium oleate (mM)	-	-	-	-	-	-	-	0.80
Maleic acid (mM)	-	-	19.10	19.10	-	-	55.00	55.00
Monobasic sodium phosphate monohydrate (mM)	28.40	28.40	-	-	-	-	-	-
Sodium chloride (mM)	106	106	68.60	68.60	203	203	126	126
Sodium hydroxide (mM)	8.70	8.70	101	101	101	101	82.00	82.00
Glacial acetic acid (mM)	-	-	-	-	144	144	-	-
pH	6.5	6.5	6.5	6.5	5.0	5.0	5.8	5.8
Osmolarity (mOsm/kg)		270		180		670		390
Buffer capacity (mM/dpH)		12		10		76		25

Table 2: Chemical properties and solubility of calcein (CAL), ibuprofen (IBP), indomethacin (IND), metoprolol (MTP), metronidazole (MTR) and naproxen (NPR).

Abbreviation	pKa	Log P	BCS class[f]	MW (g/mol)	Wavelength (nm)	Solubility (mg/mL)
CAL	1.8/9.2[a]	-1.71[b]	-	622.55	Ex.: 485 Em.: 520	- -
IBP	4.45[c]	3.97[d]	II	206.29	220	pH 5.5: 0.37 pH 6.2: 1.06 pH 7.4: 1.72
IND	4.42[c]	4.27[d]	II	357.79	254	pH 5.5: 0.03 pH 6.2: 0.09 pH 7.4: 0.71
MTP	9.56[c]	1.88[d]	I	267.36	274	pH 5.5: 1.01 pH 6.2: 1.07 pH 7.4: 0.99
MTR	2.62[e]	-0.02[d]	I	171.15	320	pH 5.5: 1.02 pH 6.2: 1.09 pH 7.4: 1.07
NPR	4.18[c]	3.18[d]	II	230.26	270	pH 5.5: 0.15 pH 6.2: 0.56 pH 7.4: 1.37

[a]: Flaten et al. 2006b
[b]: Naderkhani et al. 2014a
[c]: Avdeef 2003
[d]: Benet et al. 2011
[e]: Rediguieri et al. 2011
[f]: Amidon et al. 1995

Supplementary material

Mimicking regional and fasted/fed state conditions in the intestine with the mucus-PVPA *in vitro* model: the impact of pH and simulated intestinal fluids on drug permeability

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1. Quantification methods: drug permeated in the acceptor compartment

The quantification of the amount of drug found in the acceptor compartment at the end of the permeability study was carried out with different quantification methods according to the specific compound. Previous studies by us have assessed the possibility of interference of lipids from the PVPA barrier in the quantification process, and have concluded that no lipids were found in the acceptor compartment (Flaten et al., 2007). UV-Vis spectrophotometry was sensitive enough for the quantification of IBP, MTP, MTR and NPR in the permeation studies, as the absorbance of each specific drug found in the acceptor compartment was inside the specific standard curve range and above the LOD and LOQ values (Table 1S). However, for the quantification on IND, HPLC-UV was needed since the absorbance in the acceptor compartment was not appreciable enough by the UV-Vis spectrophotometry quantification method.

Table 1S: Parameters for the quantification of CAL, IBP, IND, MTP, MTR and NPR.

	pH	***Cal. Curve range (nmol/mL)***	***R^2***	***LOD (nmol/mL)***	***LOQ (nmol/mL)***
CAL	5.5	0.10-2.20	0.9999	0.05	0.16
	6.2	0.10-2.20	0.9998	0.07	0.22
	7.4	0.02-2.20	0.9995	0.09	0.27
IBP	5.5	8.00-150.00	0.9994	6.70	20.29
	6.2	8.00-150.00	0.9994	6.70	20.29
	7.4	8.00-150.00	0.9999	2.99	9.06
IND UV	5.5	12.00-120.00	1	1.16	3.53
	6.2	12.00-120.00	1	0.82	2.49
	7.4	12.00-120.00	0.9992	7.86	23.82
IND HPLC	7.4	0.015-30.00	0.9998	0.56	1.70
MTP	5.5	1.00-30.00	0.9991	1.86	5.65
	6.2	1.00-30.00	0.9997	1.09	3.31
	7.4	1.00-30.00	0.9992	2.08	6.32
MTR	5.5	18.00-366.00	0.9997	14.00	42.42
	6.2	18.00-366.00	0.9999	8.75	26.50
	7.4	18.00-366.00	0.9998	6.50	19.70
NPR	5.5	50.00-250.00	0.9989	20.07	60.82
	6.2	50.00-250.00	0.9993	16.72	50.67
	7.4	50.00-250.00	0.9991	18.77	56.89

For the validation of the HPLC-UV quantification method of IND, different parameters have been assessed. First, the evaluation of the right column type, mobile phase, time run and flow was carried out by injecting a standard IND solution (in PBS pH 7.4) and by monitoring the separation profile at 254 nm. The retention time of IND obtained with a Waters X-select™ CSH ™ C18 (2.5 μm, 3.0 × 75 mm) XP column, a flow rate of 0.5 mL/min and a mobile phase of acetonitrile and MilliQ water with 0.1% glacial acetic acid (60:40, v/v) was found to be 3.05 during a total run time of 5.5 minutes, while the retention time of the solvent front was found to be 1.07 minutes (Fig. 1S). The IND standard was injected at increasing concentrations (9

dilutions; 3 replicates for each dilution; 0.015-30 nmol/mL) in order to obtain a satisfactory calibration curve (R^2 = 0.9998; LOD = 0.56 nmol/mL; LOQ = 1.70 nmol/mL). The retention capacity factor k was also evaluated and found to be acceptable (k = 1.87), together with the peak asymmetry factor (A_s = 1.22) and efficiency (N = 674). As both the standard IND solution and the samples obtained from the permeability study were only containing IND, the assessment of the selectivity and resolution was not possible.

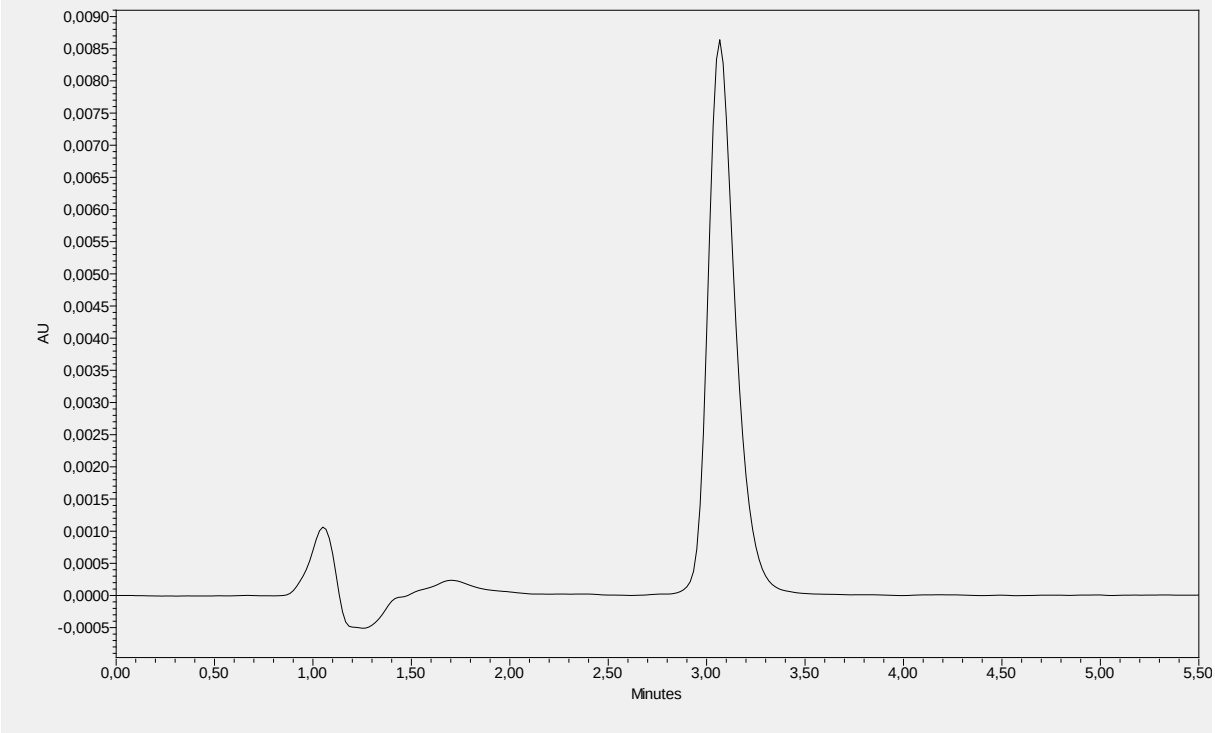

Fig. 1S: Chromatogram of IND standard.

The spectrofluorometric determination of CAL was carried out following the method described by Flaten and colleagues (2006b). The excitation and emission wavelengths (485 and 520 nm, respectively) were chosen to accurately quantify the compound and to avoid a crosstalk between excitation and emission curves. A CAL standard solution (in PBS pH 7.4) was prepared at increasing concentrations (9 dilutions; 3 replicates for each dilution; 0.02-2.2 nmol/mL) in order to obtain a suitable calibration curve (R^2 = 0.9995; LOD = 0.09 nmol/mL; LOQ = 0.27 nmol/mL).

2. Correlation between the (mucus)-PVPA and the Caco-2 model

A correlation between permeabilities obtained using a mucus-comprising Caco-2 model (Berben et al., 2018b) with the permeability of four different drugs (IBP, IND, NPR, MTP) obtained using the PVPA barriers both in the absence and presence of mucus was carried out. The Caco-2 data used for these correlations was obtained from a study where mucus was added on top of Caco-2 cells prior to the addition of the drug in solution, which was dissolved in FaHIF (fasted state human intestinal fluids) at pH 6.83 (Berben et al., 2018b). The correlations are the following:

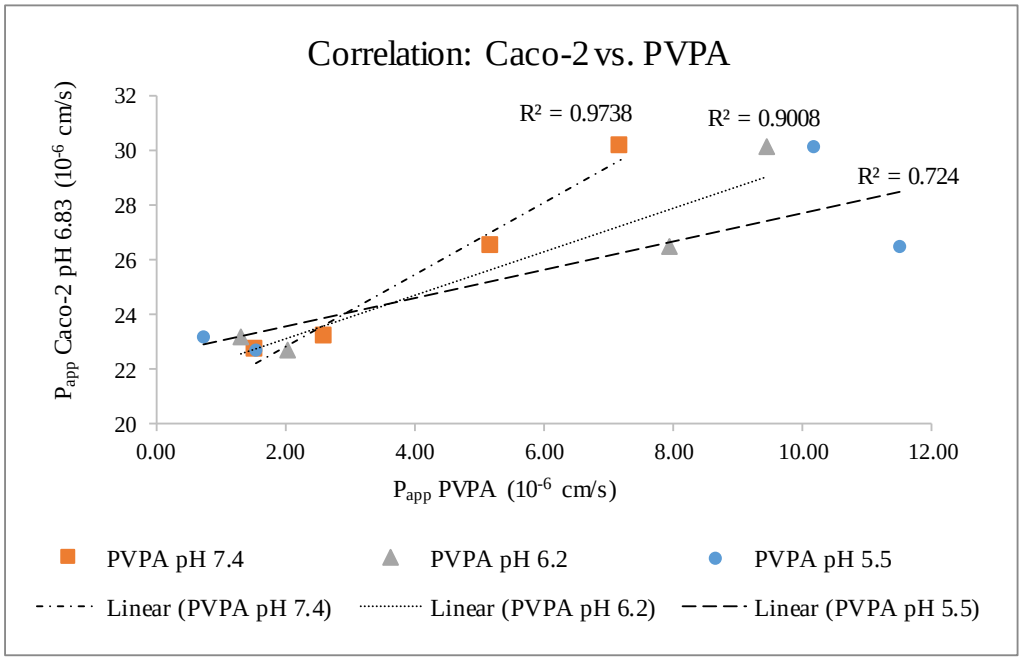

Fig. 2S: Correlation between the P_{app} obtained using the Caco-2 model (data from: Berben et al., 2018b) and the PVPA model.

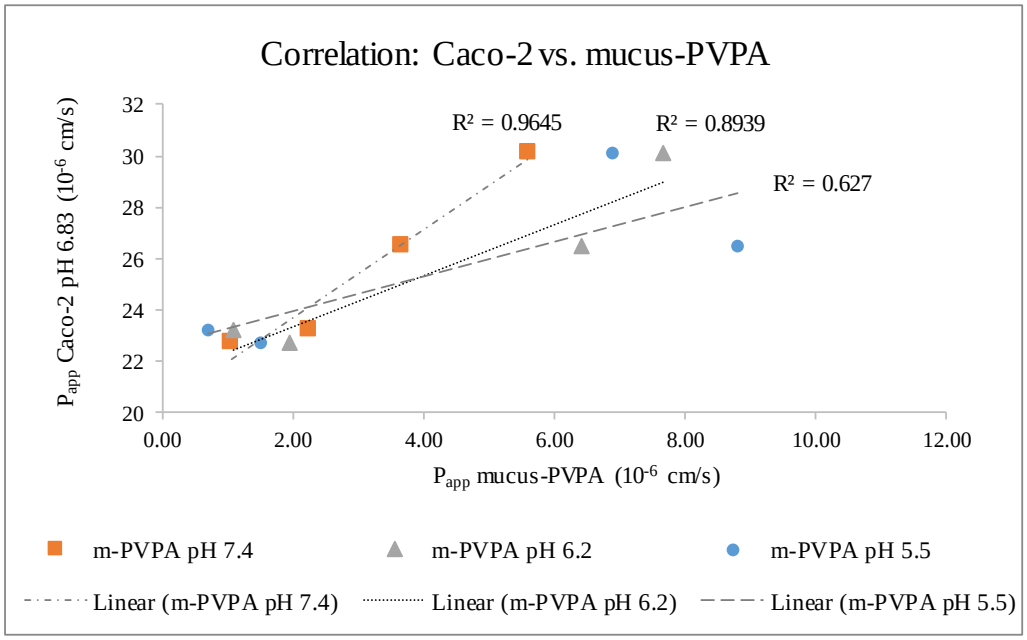

Fig. 3S: Correlation between the P_{app} obtained using the Caco-2 model (data from: Berben et al., 2018b) and the mucus-PVPA model.

As it can be observed in Fig. 2S and 3S, a satisfactory correlation for all pH conditions between the permeability data obtained using the Caco-2 model and the (mucus)-PVPA model has been obtained. This was especially evident in the case of (mucus)-PVPA data at pH 7.4, most likely due to the fact that the Caco-2 data was exclusively collected at pH 6.83. These correlations suggest the suitability of the model used in the current study for the investigation on drug permeation.