Biomimetic PVPA *in vitro* model for estimation of the intestinal drug permeability using fasted and fed state simulated intestinal fluids

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Abstract

A prerequisite for successful oral drug therapy is the drug's ability to cross the gastrointestinal barrier. Considering the increasing number of new chemical entities in modern drug discovery, reliable and fast *in vitro* models are required for early and efficient prediction of intestinal permeability. To mimic the intestinal environment, use of biorelevant media may provide valuable information on *in vivo* drug permeation. The present study aims at improving the novel biomimetic phospholipid vesicle-based permeation assay's ($PVPA_{biomimetic}$) biorelevance by investigating the applicability of the biorelevant media; fasted state simulated intestinal fluid (FaSSIF) and fed state simulated intestinal fluid (FeSSIF). The FaSSIF and FeSSIF's influence on the permeability of the model drugs acyclovir, indomethacin, griseofulvin and nadolol was then assessed. The barriers' robustness in terms of storage stability was also evaluated. The barriers were found to maintain their integrity in presence of FaSSIF and FeSSIF. The model drugs showed changes in permeability in presence of the different simulated intestinal fluids that was in agreement with previous reports. Moreover, the barriers showed improved storage stability by maintaining its integrity for 6 months. Altogether, this study moves the $PVPA_{biomimetic}$ an important step towards a better *in vitro* permeability model for use in drug development.

Key words

Liposomes, permeability, *in vitro* model, artificial membrane, lipid, simulated intestinal fluid

List of abbreviations

BCS, biopharmaceutics classification system; Chol, cholesterol; CMC, critical micelle concentration; E-80, egg phospholipids; ER, electrical resistance; FaB, fasted state buffer; FaSSIF, fasted state simulated intestinal fluid; FeB, fed state buffer; FeSSIF, fed state simulated intestinal fluid; GI, gastrointestinal tract; HPLC, high performance liquid chromatography; OrBiTo, oral biopharmaceutics tools; PAMPA, parallel artificial membrane permeability assay; PB, phosphate buffer; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PVPA, phospholipid vesicle-based permeation assay; $PVPA_{biomimetic}$, PVPA with PC, PE, PS, PI and Chol in the barrier; $PVPA_o$, PVPA with E-80 in the barrier.

1. Introduction

Although several routes of administration are available to bring a drug to the desired site of action, oral administration is still the most important and prevalent route of administration, due to its cost efficiency, convenience and patient compliance (Kanzer et al., 2010). Modern drug discovery programs are often based on combinatorial chemistry and high-throughput screening, and a majority of the new drug candidates are suffering from poor water solubility (Augustijns et al., 2014). It is recognized that up to 90 % of the new chemical entities are classified as Class II and IV in the Biopharmaceutics Classification System (BCS) (Sjogren et al., 2013). Drug compounds that are classified as Class II are suffering from poor solubility, but are highly permeable, while the class IV compounds suffer both from insufficient solubility and permeability (Dressman and Reppas, 2000). Insufficient oral bioavailability of a drug is usually associated with limited solubility and/or permeability (Flaten et al., 2011).

Consequently, appropriate and reliable high throughput *in vitro* models to assess absorption and distribution potential of new drug candidates and drug formulations early in the development process are a prerequisite for increased success rate as well as reduced time and costs (Lennernäs, 2014). Recently, approval of a new European project within the Innovative Medicines Initiative (IMI) program in the area of oral biopharmaceutics tools (OrBiTo) underlines the importance of this issue. The project aims at improving the understanding of how orally administered drugs are absorbed from the gastro intestinal tract (GI) and simultaneously apply this information to develop new advanced tools applicable in permeability screening (Moverare-Skrtic et al., 2014).

A number of *in vitro* models for the prediction of intestinal absorption, able to provide information on these aspects early in the development process, have been established. Currently, the common standard in *in vitro* permeability screening which aims at simulating the intestinal epithelia is the cell-based Caco-2 model, which has been extensively used in both academia and industry (Artursson, 1990; Artursson et al., 2001; Bergström et al., 2009). This model is of special value in estimating the impact of active transport proteins on the permeability of orally administered drugs. However, cell-based models might be too complicated, time consuming, and costly in regard to modern high-throughput requirements. In addition, the reproducibility and stability of this cell-based model might be prone to variations as the culturing cells are living systems (Gantzsch et al., 2014; Sambuy et al.,

2005). Another currently established model designed for rapid screening of drug permeability is the parallel artificial membrane permeation assay (PAMPA) (Avdeef, 2003b; Kansy et al., 1998; Wohnsland and Faller, 2001). PAMPA generally consists of a filter support covered with phospholipids in organic solvents, which allows for medium- to high-throughput screening of drug permeability. However, lack of resemblance with the biological membrane structure, few reports on use in permeability screening from different formulations and limited knowledge on use of more biorelevant media is placing a limitation on this model (Flaten et al., 2006b; Markopoulos et al., 2014).

Recently, a new addition to the phospholipid vesicle-based permeation assay (PVPA) family, the PVPA with a biomimetic lipid composition ($PVPA_{biomimetic}$), has been introduced by us for better assessment of passive intestinal permeability (Naderkhani et al., 2014). The PVPA developed for the screening of passive permeability, consists of a tight barrier of liposomes on a filter support. The $PVPA_{biomimetic}$ established to be an intestinal mimicking barrier, has proven to be tremendously more robust towards the presence of tensides and co-solvents compared to the original PVPA ($PVPA_o$), granting the model an enhanced ability to estimate the permeability of poorly soluble compounds. This is of great importance since many drug candidates today are suffering from poor water solubility (BCS class II or IV drugs) (Bergstrom et al., 2014).

Another important point in permeability estimation is to utilize more biorelevant media to mimic the *in vivo* conditions in the test set up. It is known that drugs from different classes might be affected differently in the presence of biorelevant fluids and also the presence of certain components in the GI fluid such as lecithin and bile salt (sodium taurocholate) might have a large impact on the solubility and permeability of poorly soluble compounds (Bergstrom et al., 2014; Dahan and Miller, 2012). Among the biorelevant media, FaSSIF and FeSSIF, first introduced in 1998 by Dressman and co-workers and later modified to better predict *in vivo* behavior of drugs, are the most utilized fluids originally applied as dissolution medium and later employed as medium in the permeability screening (Galia et al., 1998; Jantratid et al., 2008). FaSSIF has previously been shown to be compatible with the original $PVPA_o$-barriers consisting of egg phospholipids (Fischer et al., 2012) However, the more challenging FeSSIF has never been tested in any of the PVPA models.

The aim of this study was thus to improve the $PVPA_{biomimetics}$ biorelevance by i) investigating the applicability of the biorelevant media FaSSIF and FeSSIF in the novel $PVPA_{biomimetic}$ model and ii) evaluating FaSSIF and FeSSIF's influence on the permeability of model drugs (acyclovir, indomethacin, griseofulvin, nadolol) with a diverse set of physicochemical properties leading to classification into different BCS classes. In addition, the $PVPA_{biomimetic}$ barrier robustness in terms of the storage stability was evaluated to improve the assay's user-friendliness.

2. Materials and methods

Acetonitrile CHROMASOLV®, acycloguanosine, cholesterol (Chol), Fiske-SubbaRow reducer, phosphatidylinositol (PI), formic acid eluent additive for LC-MS, griseofulvin, glyceryl monooleate, indomethacin, maleic acid, methanol CHROMASOLV®, nadolol, potassium phosphate monobasic, sodium chloride, sodium dihydrogen phosphate, sodium hydroxide, sodium oleate, sodium taurocholate were obtained from Sigma-Aldrich, Steinheim, Germany. Ammonium molybdate, hydrogen peroxide, and di-sodiumhydrogenphosphat-dihydrat were purchased from Merck KGaA, Darmstadt, Germany. Lipoid egg phospholipids (E-80), egg phophatidylcholine (PC), egg phophatidylethanolamine (PE), egg phophatidylserine (PS) were obtained from Lipoid, Ludwigshafen, Germany. Sulphuric acid was provided by May and Baker LTD Dagenham, England. All chemicals used in the experiments were of analytical grade.

Filter inserts (Transwell®, d = 6.5 mm) and plates were purchased from Corning Inc., Corning, New York, USA. The mixed cellulose ester filters (0.65 µm pore size) were purchased from Millipore, Billerica, Massachusetts, USA. Whatman® nucleopore track-etch membrane filters (0.4 µm, 0.8 µm and 1.2 µm pore size) were obtained from Whatman, part of GE Healthcare, Little Chalfont, UK.

2.1. Preparation of liposomes for the $PVPA_{biomimetic}$ barriers

Liposomes were prepared using the film hydration method as previously described (Naderkhani et al., 2014). Briefly, two different lipid compositions were used:

1. E-80 (100 %) for liposomes to fill the pores of the filter in the first part of the preparation procedure.

2. PC (26.5 %, w/w), PE (26.5 %, w/w), PS (7 %, w/w), PI (7 %, w/w), Chol (33 %, w/w), for liposomes for the layer on top of the filter in the last part of the preparation procedure.

The lipids were dissolved in a mixture of chloroform and methanol (2:1, v/v) in a round bottom flask. The organic solvents were removed by evaporation. The dried lipid films were then hydrated with phosphate buffer (PB pH 7.4), followed by the addition of 10 % (v/v) ethanol to achieve a 6 % or 3 % (w/v) liposomal dispersion for composition 1 and 2, respectively (Flaten et al., 2006b; Naderkhani et al., 2014). The liposomes were then extruded by hand using either syringe filter holders or Lipofast (Avastin Europe GmbH, Mannheim, Germany) to obtain liposomes of different size distributions as previously described (Naderkhani et al., 2014).

2.2. Preparation of the $PVPA_{biomimetic}$ barriers

The $PVPA_{biomimetic}$ barriers, designed to study the permeability from the intestinal epithelia, were prepared according to the procedure previously described (Naderkhani et al., 2014). In brief, the procedure was as follows:

- Addition of small liposomes (extruded through 400 nm pore filter) of E-80 to fill the pores of the filter support: liposomes (100 µl) were deposited on the filter support (650 nm pores) and centrifuged at 610*g* for 5 min. The procedure was repeated with addition of liposomes (100 µl) and centrifuged for 10 min.
- Heating of barriers at 50 °C for 45 min.
- Addition of large liposomes (extruded through 1200 nm pore filter) of PC/PE/PS/PI/Chol to settle on top of the filter support: liposomes (50 µl) was added and heated at 50 °C for 30 min (procedure performed twice).
- Freezing of barriers at -70 °C for minimum 60 min.
- Thawing of barriers at 50 °C for 105 min prior to the permeability experiments.

2.3. Permeation studies using the $PVPA_{biomimetic}$ model

Permeation studies were performed using solution of model drugs or calcein in PB pH 6.2, fasted state buffer (FaB), fed state buffer (FeB), FaSSIF and FeSSIF. Preparation of FaB, FeB, FaSSIF, FeSSIF was done according to the procedure described by Jantratid and co-workers (Jantratid et al., 2008). Table 1 provides the composition of the different media and Table 2 gives an overview of the physicochemical and biopharmaceutical properties of

employed model drugs and the marker calcein. Calcein was, based on experience from previous studies, chosen as a sensitive marker to detect any changes in barrier integrity (Engesland et al., 2014; Engesland et al., 2013; Fischer et al., 2011; Flaten et al., 2009; Flaten et al., 2006a; Flaten et al., 2006b; Flaten et al., 2008; Flaten et al., 2007; Naderkhani et al., 2014). The model drugs acyclovir (3 mM), griseofulvin (0.05 mM), indomethacin (0.3 mM) and nadolol (6 mM) were dissolved in concentrations that were high enough to facilitate reliable analyses of the samples from the acceptor compartments, but still well below the saturation limits. The inserts were loaded with the different drug solutions (100 µl) and placed in acceptor wells containing PB pH 7.4 (600 µl) according to the procedures described earlier (Flaten et al., 2006b). The inserts were further moved at certain time intervals over a period of 5 h (every 1 h the first 3 h, and every 0.5 h the remaining 2 h) to new acceptor wells to maintain sink conditions. In the case of nadolol, the inserts were moved every second hour for 12 h to ensure reliable analysis of samples from the acceptor compartments. At the end of the permeation experiments, samples from each donor and acceptor compartment were analysed as described in section 2.7 employing HPLC for the model drugs and fluorescent measurement for calcein.

The apparent permeability coefficient (P_{app}) of the different formulations was calculated from the linear part of the curve obtained under steady state conditions as previously described (Flaten et al., 2006b). The electrical resistance (ER) across the barriers was further measured (Millicell-ERS, Millipore, USA) as a barrier integrity control (Flaten et al., 2006b).

Upon completion of the permeation experiments, the recovery was calculated by dividing amount of drug from the donor compartment as well as the total amount of drug from the acceptor compartments by the initial donor concentration. The calculated recoveries are given in percentage.

2.4. Loss of sodium taurocholate

In order to evaluate feasible interaction of sodium taurocholate with the PVPA barriers, the loss of sodium taurocholate from the donor compartment was determined during 5 h of incubation in the presence of both FaSSIF and FeSSIF. Also, experiments with blank samples consisting of FaB and FeB were performed. At predetermined time intervals, the samples were withdrawn from the donor compartment and concentrations determined by HPLC. The loss of sodium taurocholate (%) was calculated according to the following equation (Fischer et al., 2012):

$$Loss\ TC\ (\%) = (1 - \left(\frac{Donor}{Medium - Blank}\right) x\ 100 \quad (1)$$

where *Donor* is the concentration of sodium taurocholate in the donor samples withdrawn during the incubation, *Medium* is the concentration of sodium taurocholate in FaSSIF or FeSSIF, and *Blank* is the concentration of sodium taurocholate in FaB or FeB.

2.5. Phosphorous assay

The amounts of phospholipids released from the barriers were determined using the Bartlett method (Bartlett, 1959). Briefly, upon 5 h incubation with PB pH 6.2, FaB, FeB, FaSSIF, FeSSIF and 0.5 % Triton X-100 samples (50 µl) from the acceptor compartment were withdrawn, diluted with distilled water (50 µl), mixed with 10 N H_2SO_4 (0.5 ml) and heated in an oven at 160 °C for 3 h. The samples were then cooled to room temperature and 2 drops of 30 % (v/v) H_2O_2 were added before heating at 160 °C for additional 1.5 h. After cooling to room temperature, 22 % (v/v) ammonium molybdate (4.6 ml) and Fiske-SubbaRow reagent (0.2 ml) were added, the sample mixed and further heated for 7 min at 100 °C. All samples were analysed by UV spectrophotometer (Spectramax 190, Molecular Device Corporation, California, USA) at λ = 830 nm (Bartlett, 1959). Sample of PB only was treated in the same way as the samples containing phospholipids and used as blank.

2.6. Storage stability study

In the storage stability studies, the $PVPA_{biomimetic}$ barriers were prepared as previously described and stored for a period of 12 h (control), 1, 2, 3 and 4 weeks and 2, 4 and 6 months at -70 °C. The permeability of the hydrophilic marker calcein was used to monitor the stability and integrity of the barriers and ER across the barriers were used as integrity control (Flaten et al., 2006b).

2.7. Analytical methods

Detection of griseofulvin, indomethacin and sodium taurocholate were performed by HPLC with a Waters X-select™ CSH™ C18 (2.5µm, 3.0x75mm) XP column on a Waters e2795 Separations Module equipped with a Waters 2489 UV/Visible Detector (Waters, Milford, Massachusetts, USA). Regarding Nadolol, a faster and more reliable separation was obtained with a Waters Atlantis® T3 column (3µm, 4.6x50mm). Mobile phases, run time, flow and wavelengths for HPLC quantification are listed in Table 3.

Quantification of acyclovir was, due to a more complicated separation on HPLC, performed on a UPLC, employing a Waters Acquity UPLC® BEH C18 (1.7µm, 2.1x100mm) column on a Waters Acquity I-class UPLC with Photodiode Array Detector. The analyses were performed with the chromatographic conditions as provided in Table 4 at wavelength 253 nm. Any interference of similar retention time between components in the different media and the drugs were ruled out before the experiments were performed.

Fluorescence measurements of calcein were performed as previously described using a Polarstar fluorimeter (Fluostar, BMG Technologies, Offenburg, Germany) with excitation and emission wavelengths at 485 and 520 nm, respectively (Flaten et al., 2006b).

2.8. Statistical evaluation

To identify significant differences between two sets of data, the Student's *t*-test for the comparison of two means were performed. A significant level of $p < 0.05$ was consistently used.

3. Results and discussion

One of the major issues in drug development today is, as already stated, that drug candidates are often suffering from poor water solubility which again could be the rate limiting step in drug absorption. An important point for a better prediction of a drug's performance *in vivo* is thus to use the biorelevant media to mimic the *in vivo* condition in the permeability test set up (Bergstrom et al., 2014). The $PVPA_{biomimetic}$ model was selected as an *in vitro* model to better predict the GI absorption in the presence of FaSSIF and FeSSIF. This is, to the best of our knowledge, the first time the PVPA models were challenged by assessing the permeability in the presence of FeSSIF. The $PVPA_{biomimetic}$ has shown to be substantially more robust towards tensides and co-solvents compared to the original $PVPA_o$ and is thus more suitable to withstanding the harsh conditions in FeSSIF.

3.1. Barrier integrity in the presence of FaSSIF and FeSSIF

In order to evaluate the compatibility of $PVPA_{biomimetic}$ barriers with the biorelevant media (FaSSIF and FeSSIF), the permeability experiments were conducted using the hydrophilic marker calcein in PB pH 6.2, FaB, FaSSIF, FeB and FeSSIF. The bile salt sodium taurocholate, present in both FaSSIF and FeSSIF in different concentrations, is known to interact with the membrane lipids at low concentrations (Jantratid et al., 2008; Schubert et al., 1986). PB pH 6.2, FaB and FeB, which do not contain the bile salt, were therefore included to

compare the barrier integrity and further evaluate any possible interaction of FaSSIF and FeSSIF with the $PVPA_{biomimetic}$ barrier. FaSSIF has previously shown to be compatible with the original $PVPA_o$-barriers. Non-significant changes in calcein permeability was here shown to give no visual evidence of structural changes from confocal micrographs (Fischer et al., 2012). The $PVPA_{biomimetic}$ has earlier shown to be compatible with different tensides as well as being more robust in presence of Triton X-100 compared to the original $PVPA_o$ barrier (Fischer et al., 2012; Naderkhani et al., 2014). Calcein permeability in the presence of Triton X-100 was therefore included in Figure 1 for comparison (Naderkhani et al., 2014).

The calcein permeability in the presence of FeSSIF (Figure 1) exhibited no significant difference compared to the permeability in PB. However, due to a significant decrease ($p < 0.05$) in calcein permeability in the presence of FeB compared to PB the calcein permeation significantly increased ($p < 0.05$) in the presence of FeSSIF compared to FeB. The high bile salt concentration in FeSSIF might have contributed to the increased calcein permeability by possibly inducing minor disruptions of the barrier. However, the increase in calcein permeability in the presence of FeSSIF was lower than for Triton X-100 earlier found compatible with the model (Naderkhani et al., 2014). FaB and FaSSIF both caused a significant increase in calcein permeability compared to PB. There were however no significant differences in calcein permeability when FaB and FaSSIF were compared. These findings are in agreement with earlier studies, performed with the original $PVPA_o$ by Fischer and co-workers (Fischer et al., 2012). In addition, the ER values remained within the limits (1000-3000 ohm/insert) for the barriers to pass the integrity control for all the tested media (Flaten et al., 2006b; Flaten et al., 2008). Our findings thus indicates that the $PVPA_{biomimetic}$ is compatible in the presence of FaSSIF and FeSSIF in respect to both calcein permeability and ER across the barriers, and our results are in full agreement with earlier studies employing FaSSIF in the $PVPA_o$ (Fischer et al., 2012).

The use of FaSSIF and FeSSIF in other permeability models such as the Caco-2 cell model has also been reported and it was shown that the presence of these media (in original or modified form) neither affected the cell viability or the barrier integrity (Fossati et al., 2008; Ingels et al., 2004; Ingels et al., 2002; Kataoka et al., 2006; Markopoulos et al., 2014; Patel et al., 2006). On the other hand, regarding the PAMPA model the knowledge of the effect of biorelevant media is very limited (Markopoulos et al., 2014). This further highlights the PVPA models' advantages in drug permeability screening.

3.2. Phospholipids released from the $PVPA_{biomimetic}$ barrier

To further confirm the compatibility of the $PVPA_{biomimetic}$ barrier with FaSSIF and FeSSIF, as well as provide valuable additional information regarding the barrier integrity, the amount of phospholipids released from the $PVPA_{biomimetic}$ barrier during the experiments were determined. The data indicate a slight, but non-significant increased release of phospholipids during the incubation with FaSSIF and FeSSIF compared to PB, FaB and FeB (Figure 2). Moreover, 0.5 % Triton X-100, used as a reference, induced a release of approximately 40 µg phospholipids, which is a significant ($p < 0.05$) two-fold increase in the release compared to FaSSIF and FeSSIF. However, even though Triton X-100 showed an increased release of phospholipids from the membrane compared to the control, these data demonstrates a more robust model compared to the original $PVPA_o$ which exhibited highly elevated levels of phospholipids (approximately 900 µg) in the donor chamber upon incubation with Triton X-100 (Fischer et al., 2012). This denotes a 20-fold decrease in released phospholipids as compared with the $PVPA_o$, demonstrating a tremendous increase in integrity and stability of the $PVPA_{biomimetic}$ barrier. Further, when comparing our results with earlier published data on the $PVPA_o$ we observed a 10-fold reduction in the release from the barrier in presence of FaB and FaSSIF compared with the $PVPA_o$ (Fischer et al., 2012). Previously it has been reported that the lipids Chol, PS and PE stabilize the membrane against the action of sodium taurocholate to different degrees (Schubert and Schmidt, 1988). Subsequently, the presence of these lipids in the $PVPA_{biomimetic}$ barrier appears to have contributed to stabilization of the membrane against the aggressive surfactant, sodium taurocholate. These findings confirm that the $PVPA_{biomimetic}$ is stable in the presence of FaSSIF and FeSSIF, and supplements our conclusion that the barrier integrity, according to calcein permeability and ER, is maintained.

3.3. Loss of sodium taurocholate from the donor

The bile salt, sodium taurocholate, is present in FaSSIF and FeSSIF in concentrations of 3 mM and 10 mM, respectively (Jantratid et al., 2008). The interaction between sodium taurocholate and unilamellar vesicles has earlier been studied and according to Schubert and co-workers low bile salt concentration appears to bind to the vesicles and interact with several membrane lipids, and particularly with lecithin (Schubert et al., 1986; Schubert and Schmidt, 1988). In order to elucidate any feasible interaction between sodium taurocholate and the $PVPA_{biomimetic}$ barriers we investigated the loss of sodium taurocholate during 5 h of incubation in presence of FaSSIF and FeSSIF. The results shown in Table 5 indicate that sodium taurocholate both in FaSSIF and FeSSIF is lost during the experiments, reaching a

plateau at approximately 30 % loss after 4 h of incubation. This finding indicates that approximately 30 % of sodium taurocholate is possibly bound to or incorporated within the PVPA barrier. Our observation correlates well with earlier studies performed on $PVPA_o$, indicating a loss of approximately 30 % sodium taurocholate after 4 h of incubation (Fischer et al., 2012). However, the loss of sodium taurocholate from FaSSIF at the different time intervals exhibited no significant differences compared to FeSSIF which is in accordance with earlier studies suggesting that sodium taurocholate binding to the membrane is hampered as the bile salt concentration increases, probably due to bile salts interacting with each other (Nichols, 1986; Schubert et al., 1986). This is also in agreement with the release of phospholipids; no significant differences in the release of phospholipids from the barriers when comparing the presence of FaSSIF and FeSSIF were observed (Figure 2).

3.4. Influence of FaSSIF and FeSSIF on permeability of drugs

The bile salt concentration and the amount of lecithin, primarily phosphatidylcholine, are known to be important factors in the intestinal fluid affecting the drugs' solubility as well as permeability (Dahan and Miller, 2012; Jantratid et al., 2008). The intestinal solubility remains as one of the most important parameters in order to achieve the desired systemic drug concentration. It is also known that different drugs interact uniquely with physiological surfactants, depending on the physicochemical properties of both entities. Physiological surfactants in the GI tract, act to incorporate lipophilic compounds into mixed micelles consisting of bile salts and phospholipids (Augustijns et al., 2014). This effect generally results in enhanced solubility of poorly soluble drugs. The assessment of the permeation ability of the model drugs were therefore performed employing drug solutions in PB pH 6.2, FaB, FaSSIF, FeB and FeSSIF and the results from the different media were compared to elucidate their effects on drug permeation (Figure 3).

In FaSSIF the bile salt concentration is below its critical micelle concentration (CMC), thus expecting less effect on the apparent permeability of drugs compared to in FeSSIF, where the bile salt concentration is above the CMC. Due to the high bile salt concentration, FeSSIF is reported to often lead to decreased apparent permeability of drugs (Dahan and Miller, 2012; Jantratid et al., 2008; Miller et al., 2011). Another factor that may influence the apparent permeability of the drug compounds is the relation between the drugs pKa values and the pH of the different media. One has thus to consider that the intestinal permeability along the GI tract could be pH dependent, since pH might influence the drugs' charge state, and further affect the drug absorption in a more complex manner (Dahan et al., 2010; Lennernäs, 2014).

In the case of the highly lipophilic drug griseofulvin, there was a declining permeability with increased lipid and bile salt concentration in the media. Since griseofulvin is a nonionizable drug, the decreased permeability is most probably due to the drug associated within the micelles thereby decreasing the free fraction of the drug. The following rank order of permeability: PB > FaSSIF > FeSSIF has also been reported for other BCS II drugs (Markopoulos et al., 2014). Similar results for griseofulvin obtained using the $PVPA_o$ model has also been reported (Fischer et al., 2012).

The highly lipophilic drug indomethacin shows significantly increased apparent permeability in FeSSIF compared to FaSSIF opposite to what was expected according to micellar incorporation. This type of behaviour for other class II drugs has however also previously been reported by Kataoka and co-workers (Kataoka et al., 2006). They assessed the effect of FaSSIF and FeSSIF on drug permeation across Caco-2 monolayers and both danazol and albendazole (class II drugs) showed higher permeability values in the presence of FeSSIF than with FaSSIF.

The increase in recovery as shown in section 3.5 could also be a contributing factor to these results. In the case of acyclovir, which is an amphoteric compound (pKa 9.23 and 2.34) no significant changes in the apparent permeability was observed in FeSSIF compared to FaSSIF. This is as expected since drug compounds belonging to the BCS III, like acyclovir, are less likely to undergo an interaction with colloidal systems (Markopoulos et al., 2014). Consequently, the effect of pH on the apparent permeability is the most important factor for these drug compounds. However, as it is assumed that acyclovir remains neutral in our experiments as the pH in the different media is more than 3 pH units away from both its pKa values, no significant changes in the apparent permeability is expected. Nadolol exhibited significantly decreased apparent permeability both in FeB and FeSSIF compared to PB, FaB and FaSSIF. This apparent discrepancy in reduced permeability in FeB and FeSSIF, which is unexpected for a class III drug, may possibly be explained by the pH differences between the fasted and fed state media. Maleic acid, which is a component in both FaSSIF and FeSSIF, has pKa values of 1.9 for the first deprotonation and 6.1 for the second deprotonation (Prabhakaran et al., 2008). The pKa value of 6.1 gives a variation of the -1 and -2 charge states of maleic acid between fed and fasted states, which might have high impact on salt formation between maleic acid and the basic nadolol (Roche, 2007). The assumption of salt formation might elucidate the differences in apparent permeability values. Moreover, earlier studies reported by de Castro et al. (2001), confirm that the nadolol's affinity for the micelles are increasing at higher cholat concentration, which could be a possible explanation for the

decreased permeability observed for FeSSIF. It should also be mentioned that the permeability of nadolol in presence of PB is corresponding to excellent *in vivo* absorption according to previous introduced classification system by us (Flaten et al., 2006b; Naderkhani et al., 2014). Nadolol has on the other hand a fraction absorbed *in vivo* at 35%, so this is clearly a misclassification. However, by using the fed state media the permeability of nadolol falls into the correct category of moderately absorbed drugs. This might indicate that use of biorelevant fluids in the $PVPA_{biomimetic}$ could lead to better estimation of *in vivo* permeability of drugs.

3.5. Recovery rate of the model compounds

The recovery rates of the different model drugs were calculated upon the completion of the permeability studies, and the results are displayed in Table 6. The results exhibit a high recovery rate (mostly above 80 %) in the case of nadolol and acyclovir; on the contrary, the more lipophilic drugs indomethacin and griseofulvin, exhibited lower recovery rates (below 35 %) in PB, FaB and FeB. The low recovery of lipophilic drugs has earlier been reported to be due to the drug accumulation in the lipophilic barrier and/or drug adsorption to the experimental materials (Ingels et al., 2004). These effects may contribute to a physical loss of the drug, resulting in a reduced concentration in the donor compartment (driving force) again resulting in decreased permeability values (Deferme et al., 2008). Indomethacin and griseofulvin showed increased recovery rates in the presence of FaSSIF and FeSSIF indicating the media's contribution in preventing the drug adsorption to the experimental materials and/or entrapment of the compounds into the membrane. Similar observations regarding the impact of the simulated intestinal fluids on the recovery have been reported earlier (Fischer et al., 2012; Fossati et al., 2008; Ingels et al., 2004). According to those reports, the lipophilic drugs with moderate to high log *p* values (above 2.5) showed increased recovery in the presence of simulated intestinal fluids as compared to the buffers, in agreement with our findings.

3.6. Evaluation of the $PVPA_{biomimetic}$ storage stability

Considering the modern high-throughput requirements, it is important that the *in vitro* models reveal good storage stability to enable efficient permeability screening over prolonged period of time.

The storage stability of the $PVPA_{biomimetic}$ barriers was determined during the storage at – 70 °C for a period of up to 6 months. The storage duration and permeability values of the

hydrophilic marker calcein, used to assess alterations in the membrane integrity, are given in Figure 4. The results exhibit negligible changes in the permeability values during the storage from 12 h to up to 2 months. For the barriers stored from 4 to 6 months, a trend in decreased permeability with increased time indicating tighter barriers was observed. However, the changes in the permeability values compared to the control were not significant. The same trend with decreasing calcein permeability indication increased tightness of the barriers over time has also been seen for the $PVPA_o$ (Flaten et al., 2006a). The reason for this observation might be increasing dehydration of the barriers when stored for longer time.

These results indicate acceptable membrane stability under these storage conditions for the whole storage period of 6 months. This is a tremendous improvement in stability in comparison with the $PVPA_o$ barrier, which are stable for up to 2 weeks (Flaten et al., 2006a). Hence, we assume that the inclusion of Chol might have an essential impact on the stability of the $PVPA_{biomimetic}$ barrier. This effect has also been observed for the Chol containing PVPA mimicking *stratum corneum* (Engesland et al., 2014). Additional studies with other markers and drugs to further elucidate the barrier stability should be performed to enable a prolonged time period of use after the original barrier preparation. However, the barriers' increased robustness in terms of storage stability is indeed improving the assays user-friendliness and high throughput potential.

3. Conclusions

The $PVPA_{biomimetic}$ barrier was found to maintain its integrity in the presence of the biorelevant media FaSSIF and FeSSIF. This confirms the applicability of these media in the $PVPA_{biomimetic}$ model enabling a closer intestinal mimicking environment in the permeability estimations. The permeability of BCS class II and III drugs were further differently affected in the presence of the biorelevant media, in agreement with previous reports. Moreover, the $PVPA_{biomimetic}$ showed increased storage stability by maintaining its integrity for a period of 6 months compared to 2 weeks for the original $PVPA_o$. The $PVPA_{biomimetic}$ has thus shown to be more stable than the original $PVPA_o$ in terms of maintaining integrity both in the presence of biorelevant media as well as during storage, moving the PVPA model an important step forward and making it an even more relevant choice for the permeability screening in drug development.

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Conflict of interest: The authors declare that they have no conflicts of interest to disclose.

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Figure legends

Figure 1. Apparent permeability (P_{app}) of calcein (bars) and ER measured across the barriers (markers) in presence of PB pH 6.2, FaB, FaSSIF, FeB, FeSSIF and 0.5 % Triton X-100 pH 7.4 in the donor compartments. Bars and markers represent the mean ± S.D. (*n*=3).†From Naderkhani et al. (2014). *significant difference ($p \leq 0.05$) compared to PB pH 6.2 (control).

Figure 2. Amount of phospholipids released from the $PVPA_{biomimetic}$ barrier to the donor chamber upon 5 hours incubation with PB pH 6.2, FaB, FaSSIF, FeB, FeSSIF and 0.5% Triton X-100 (reference). Bars represent the mean ± S.D. (*n*=3). *significant difference ($p \leq 0.05$) compared to PB pH 6.2 (control).

Figure 3. Apparent permeability (P_{app}) of model drugs in PB pH 6.2, FaB, FaSSIF, FeB and FeSSIF. Bars represent the mean ± S.D. (*n*=3). *significant difference ($p \leq 0.05$) compared to PB pH 6.2 (control).

Figure 4. Storage stability testing of the $PVPA_{biomimetic}$ barrier according to calcein permeability (bars) and ER across the barrier (markers). Bars and markers represent the mean ± S.D. (*n*=3).

Table 1. Composition, pH and osmolality of the different media.

	PB[a]	FaB[b]	FaSSIF[b]	FeB[b]	FeSSIF[b]
Sodium taurocholate (mM)	_		3		10
Lecithin (mM)	_		0.2		2
Glyceryl monooleate (mM)	_		_		5
Sodium oleate (mM)	_		_		0.8
Maleic acid (mM)	_	19.12	19.12	55.02	55.02
di-sodium hydrogen phosphate (mM)	17.6	_	_	_	_
Sodium chloride (mM)	145	68.62	68.62	125.5	125.5
Sodium dihydrogen phosphate (mM)	18.1	_	_	_	_
Sodium hydroxide (mM)	q.s.	34.8	34.8	81.65	81.65
pH	6.2	6.5	6.5	5.8	5.8
Osmolality (mOsm kg^{-1})	_	_	180 ± 10	_	390 ± 10

[a] The composition of PB pH 6.2 is obtained from Ph.Eur. (5th edition)Ph.Eur. (5th edition)Ph.Eur. (5th edition)Ph.Eur. (5th edition)Ph.Eur. (5th edition)Ph.Eur. (5th edition).

[b] The composition of the different media are obtained from Jantratid et al. (2008).

Table 2. Physicochemical and biopharmaceutical properties of model drugs and marker.

Drug	M_w	pKa[a]	Log P[b]	Log D[a,c]	F_a (%)[d]	BCS[e]
Acyclovir	225.2	9.23, 2.34	-1.74	-1.86	20	III
Griseofulvin	352.8	-	2.18	2.18	irregular	II
Indomethacin	357.8	4.42	3.1	0.93	100	II
Nadolol	309.4	9.7	0.71	0.68	35	III
Calcein	622.5	1.8/9.2	-1.71			

[a,b] The values are from Avdeef (2003a), Stahl et al. (2011) and Zhu et al. (2002)
[c] The values are from Sugano et al. (2001) and Zhu et al. (2002).
[d] The values are from Yazdanian et al. (1998).
[e] The BCS are from Varma et al. (2012).

Table 3. Chromatographic conditions for HPLC analyses.

Compound	Elution conditions	Flow (ml/min)	Run time (min)	λ (nm)
Griseofulvin	Isocratic 55/45 water/acetonitrile[a]	0.5	6	291
Indomethacin	Isocratic 40/40/20 acetonitrile/water[b]/methanol	0.5	10	240
Nadolol	0-10 min gradient starting with 95/5 with a linear change to 5/95 water[a]/acetonitrile[a]	0.5	10	260
Sodium-taurocholate	0-2 min 100/0, 2-10 min linear change to 5/95 Water[a]/acetonitrile[a]	0.5	10	200

[a] Mobile phases contained 0.1 % (v/v) formic acid.

[b] Mobile phase contained 0.5 % (v/v) phosphoric acid.

Table 4. Chromatographic condition for UPLC analysis of acyclovir.

Time (min)	Flow (ml/min)	Solvent A[a]	Solvent B[a]	Solvent C[a]
1.0	0.6	98	2	
2.0	0.6	95	5	
3.0	0.4	20	30	50
5.0	0.4			100
8.0	0.4			100

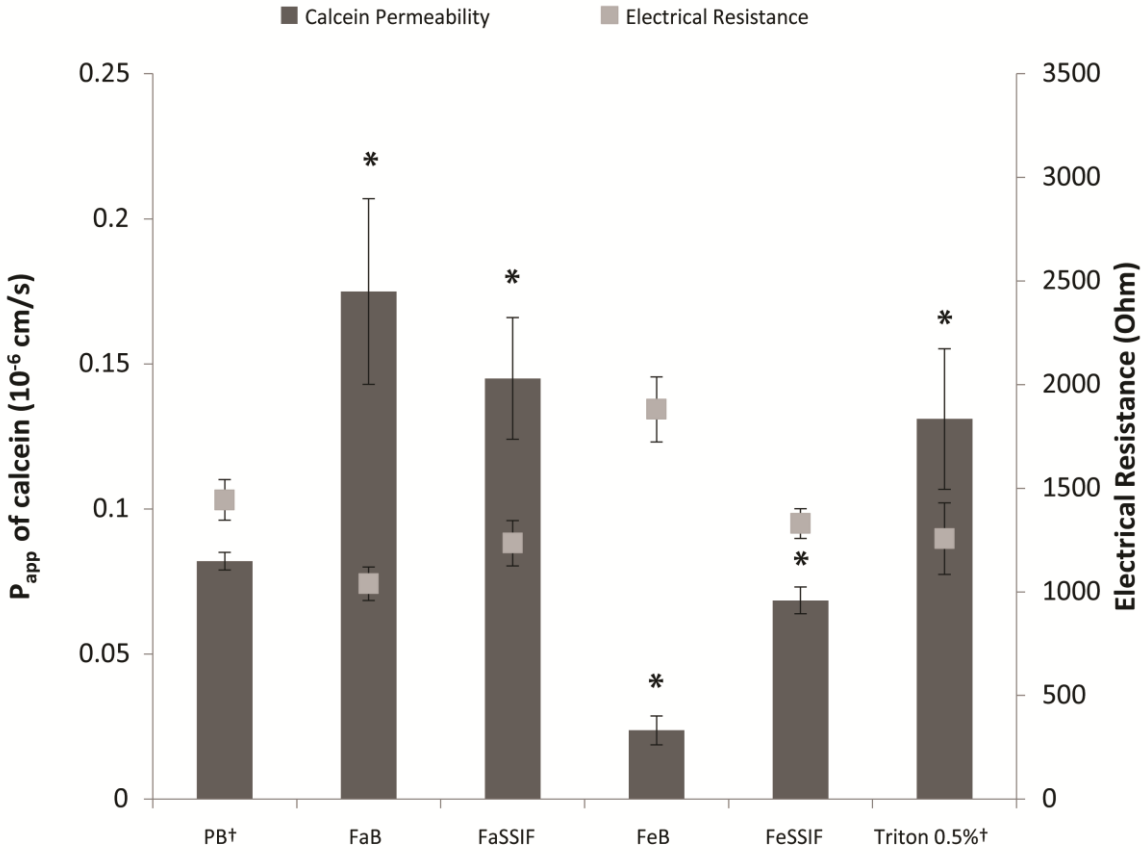
[a] 0.1 % (v/v) formic acid in water (A), 0.1 % (v/v) formic acid in acetonitrile (B), and isopropanol (C).

Table 5. Loss of sodium taurocholate from the donor during 5 h of incubation with FaSSIF and FeSSIF. Values are provided as mean ± S.D. (*n*=3).

	Loss of sodium taurocholate Mean ± S.D. (%)	
Time (h)	FaSSIF	FeSSIF
1	11 ± 2	7 ± 3
2	18 ± 1	17 ± 2
3	23 ± 1	26 ± 3
4	27 ± 2	29 ± 3
5	27 ± 2	29 ± 2

Table 6. Recovery of the model compounds in the different media. Values are provided as mean ± S.D. (*n*=3).

Medium	Recovery (%)			
	Indomethacin	Griseofulvin	Nadolol	Acyclovir
PB	24 ± 3	31 ± 3	85 ± 3	97 ± 2
FaB	25 ± 3	33 ± 2	97 ± 2	95 ± 3
FaSSIF	30 ± 1	45 ± 2	87 ± 1	86 ± 3
FeB	34 ± 2	25 ± 4	94 ± 2	92 ± 1
FeSSIF	39 ± 2	38 ±1	80 ± 1	70 ± 2



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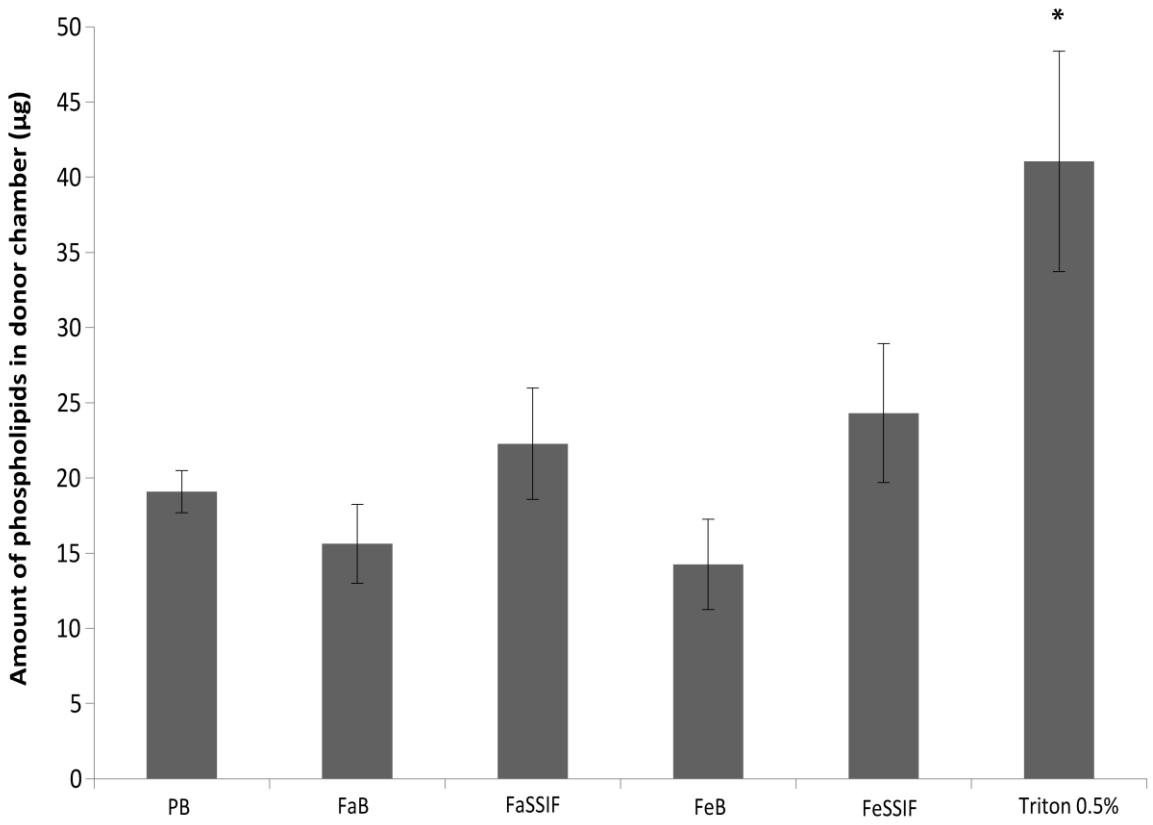

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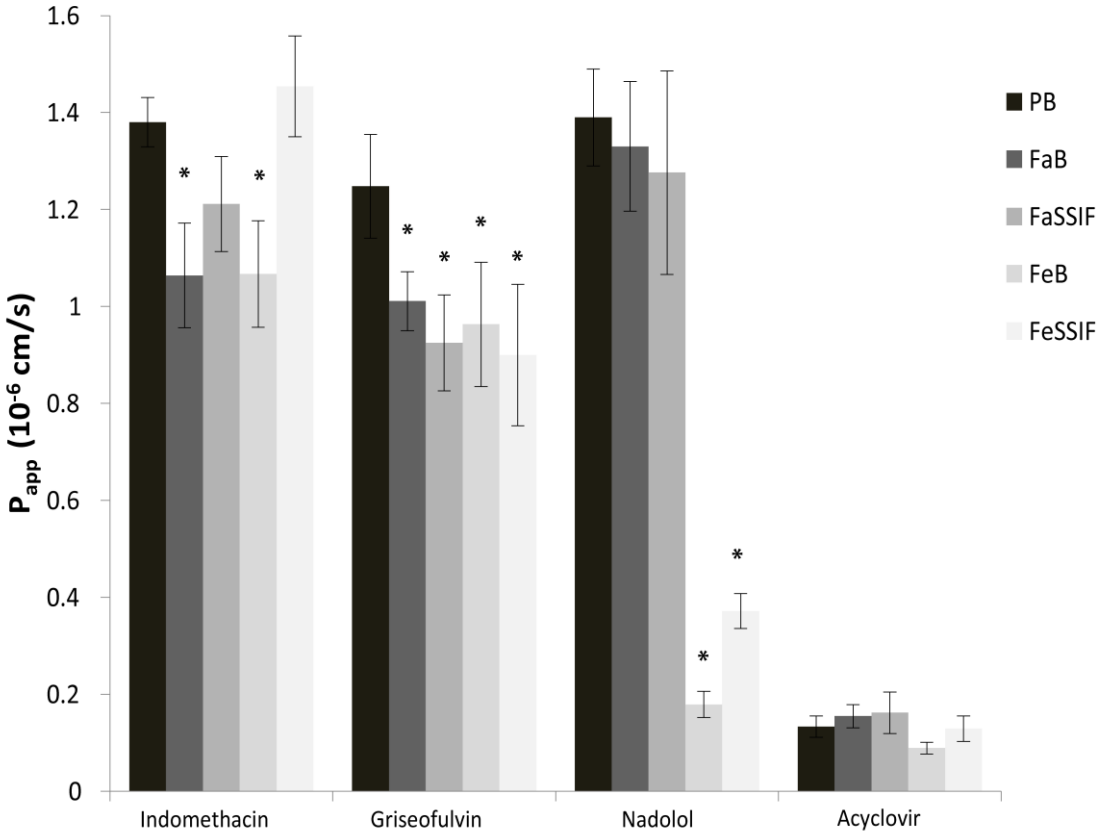
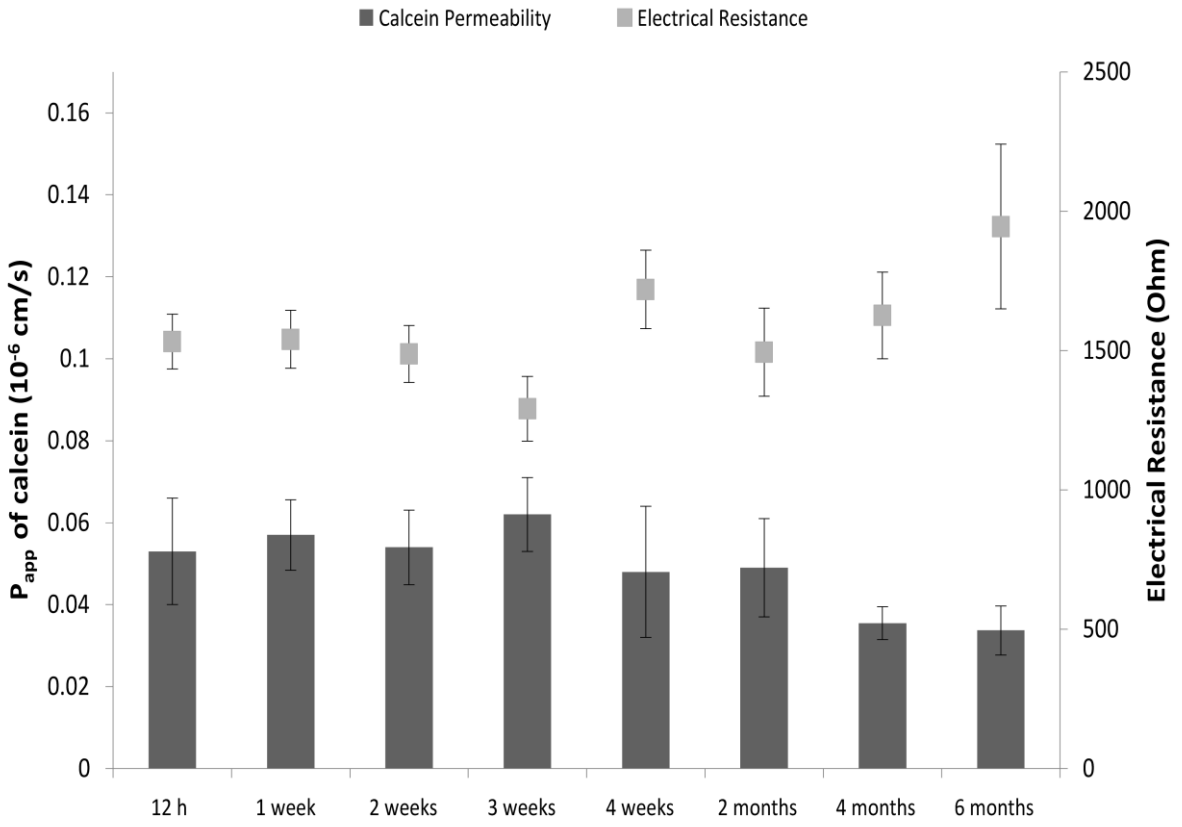


Figure 3. Apparent permeability (P_{app}) of model drugs in PB pH 6.2, FaB, FaSSIF, FeB and FeSSIF. Bars represent the mean ± S.D. (*n*=3). *significant difference ($p \leq 0.05$) compared to PB pH 6.2 (control).



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