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Use of Permeapad® for prediction of buccal absorption: A comparison to *in vitro*, *ex vivo* and *in vivo* method

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ABSTRACT

The present work explores the usefulness of Permeapad® for prediction of buccal absorption. Permeability studies with the model drug metoprolol were carried out using the Permeapad® barrier at pH values 7.4; 8.5; 9.0, and 9.5. It was confirmed that Permeapad® can withstand these conditions, and as expected, a clear increase in permeability was found with increasing pH. The permeation results across Permeapad® were compared to published *in vitro*, *ex vivo* and *in vivo* studies for the same formulations. Results showed that the permeability of metoprolol using the Permeapad® barrier correlated very well to both *in vitro* and *ex vivo* studies, ($r^2 = 0.98$ and 0.97), respectively. Furthermore, excellent *in vitro in vivo* correlation *IVIVC* ($r^2 = 0.98$) was obtained when comparing apparent permeability coefficient to the absolute bioavailability of metoprolol administered buccally to mini-pigs. Results indicate that Permeapad® can be used to mimic the buccal absorption of metoprolol as a faster and less laborious method as compared to any of the other mentioned methods.

1. Introduction

Oral administration of drugs is the most common route for drug delivery. It is associated with high patient compliance and low manufacturing costs. However, oral administration of some drugs may be seriously restricted in bioavailability due to first pass metabolism (de Vries et al., 1991), which potentially could be circumvented by administration through the buccal mucosa.

The buccal mucosa is a highly vascularized tissue, which allows fast drug uptake and can been used for both local and systemic drug delivery (Harris and Robinson, 1992). Permeation across the buccal mucosa has a number of advantages, including avoidance of enzymatic and acidic degradation of the drug in the gastro intestine (GI-tract).

The physiochemical properties of the drug substance and in particular its permeation across the oral mucosa is important for the development of a buccal formulation. Various models to determine the permeability of new active pharmaceutical ingredients (API) across the buccal mucosa have been described, where buccal mucosa tissue from porcine is used in several *ex vivo* studies (Giannola et al., 2007; Lestari et al., 2009). Even though *ex vivo* studies have shown good *in vivo in vitro correlation* (*IVIVC*) the methods are time consuming and costly (Holm et al., 2013). A cell culture line from human squamous carcinoma cell, TR146, has also shown to be able to mimic the transport properties of the buccal mucosa (Jacobsen et al., 1995; Nielsen and Rassing, 1999). However, as a common issue for cell culture studies, variations are in many cases seen between different cell batches, not to mention the long cultivation period required. Therefore a more reliable and time efficient method for screening of drug compounds for buccal permeability would be highly desired. Permeapad® is a new biomimetic assay for drug permeability screening, which has shown to be a reliable tool to investigate drug permeability related to oral absorption (Bibi et al., 2015; di Cagno et al., 2015), but the model has never been evaluated for its ability to predict buccal permeability.

Holm and co-workers have previously investigated the permeability of metoprolol, which is of high permeability (Dahan et al., 2009; Kim et al., 2006), as a function of vehicle pH using both TR146 cells and *ex vivo* porcine buccal mucosa (Holm et al., 2013) and linked these to *in vivo* data obtained from Göttingen minipigs. The aim of the present study was to investigate the permeability across Permeapad® of the same metoprolol formulations at the same pH values as in the mentioned study, and to compare these results with literature data in order to evaluate the possibility of using the Permeapad® in buccal absorption studies.

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2. Materials and methods

2.1. Materials

Hydrocortisone and metoprolol tartrate were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Soy phosphatidylcholine (PC) S-100 was a generous gift from Lipoid GmbH (Ludwigshafen, Germany). Sodium dihydrogen phosphate dihydrate ($[NaH_2PO_4]\cdot 2H_2O$), di-sodium hydrogen phosphate dodecahydrate ($[Na_2HPO_4]\cdot 12H_2O$), sodium tetraborate decahydrate ($[Na_2B_4O_7]\cdot 10H_2O$), sodium hydroxide (NaOH), hydrochloric acid (HCl) and sodium chloride (NaCl) used for the different buffers were all were obtained from Sigma-Aldrich. Water used for all the experiments was obtained from a MilliQ purification system.

2.2. Methods

2.2.1. Preparation of biomimetic barrier

The Permeapad® (Certificate No. 014557268) barrier was prepared as previously described (Bauer-Brandl and di Cagno, 2014) using soy phosphatidylcholine S-100 as the lipid layer. In brief, a thin layer of lipid was applied to a hydrophilic support sheet (Pütz GmbH, Taunusstein, Germany) in organic solution. The solvent was allowed to evaporate and the barrier formed. The final barrier therefore consisted of support layer and lipid layer. All Permeapad® barriers employed in this work were stored at room temperature protected against sunlight.

2.2.2. Functional stability pH studies on Permeapad®

In order to determine Permeapad®'s functional resistance to high pH values, permeation experiments were carried out at pH values 8.5, 9.0, 9.5, and 10 in Franz cells. An inverse permeability setup was used, hence, the donor compartment (lower chamber) was filled with 8 mL of a 1 mg/mL suspension of hydrocortisone (HC). HC was used as a model drug due to its pH independent permeability. A saturated suspension of HC was used in order to maintain a high and constant donor concentration and sink conditions (≪10% of donor concentration). The HC concentration in the donor compartment was determined prior to the experiments (*i.e.* HC solubility; 324.5 μg/mL). The acceptor compartment (upper chamber) was filled with 1.5 mL 75 mM isotonic PBS, pH = 7.40 ± 0.05; osmolality = 285 ± 5 mOsm/kg. The Permeapad® was placed between the donor and acceptor compartment and the flux (J) of HC was investigated. The permeability studies were conducted over 5 h at 25 °C and samples of 500 μL were withdrawn from the acceptor chamber every 30 min. Sample volumes were replaced with fresh buffer, and the samples were analysed using UV-VIS spectroscopy on a Genesis 10 UV/VIS (Thermo Electron Corporation, Cambridge, UK) at 248 nm. The obtained apparent permeability coefficients were compared to the control experiment, carried out at pH 7.4, using the empty barrier support.

2.2.3. Preparation of metoprolol solutions for permeability studies

0.1 mM metoprolol solutions were prepared at the following pH values: 7.4, 8.5, 9.0, and 9.5. Buffers of two different concentrations (25 mM and 100 mM) were prepared with borate (BBS) for pH of 8.5, 9.0, 9.5, and phosphate (PBS) for pH 7.4, to obtain metoprolol solutions at these pH values. The exact pH values of the various solutions were adjusted using NaOH or HCl respectively, before the addition of the drug substance, and the pH was controlled after it's addition. The buffer solutions were adjusted to isotonic properties 285 ± 5 mOsm/kg, by adding NaCl (Osmolality measured by Semi-Micro Osmometer K-7400, Herbert Knauer GmbH, Berlin, Germany).

2.2.4. Permeability studies with metoprolol solutions employing Permeapad® and support sheet, respectively

The permeability studies were carried out in the side-by-side diffusion chamber set-up (Ussing chambers, SES GmbH-Analysesysteme, Bechenheim, Germany), due to better stirring and heated water jackets in both the donor and the acceptor compartment. Both the donor and the acceptor chamber had a volume of 5 mL and exposed a surface area of the Permeapad® barrier of 1.77 cm^2. The donor chamber was filled with the respective 0.1 mM metoprolol solutions of different pH values, while the acceptor chamber contained isotonic 100 mM PBS (pH 7.4 ± 0.05) in all the experiments, in order to resemble the physiological absorption compartment. The permeability studies were carried out over 4 h at 36 °C. Samples of 500 μL were withdrawn from the acceptor chamber after 15, 30, 45, 60, 90, 120, 180 and 240 min and immediately replaced by fresh buffer. After the experiment, a 500 μL sample from the donor compartment was withdrawn and analysed in order to calculate the drug recovery. The pH values of the acceptor and donor solutions were measured prior to and after the permeation experiments. A set of further experiments were carried out in the same way, employing the empty barrier support instead of Permeapad®. Samples were analysed using a HPLC-UV method (2487 Dual Absorbance, Waters, USA). Separation was achieved using a reverse phase Acclaim C18 column (150 mm × 4.6 mm i.d., 3 μm particle size, Thermo Fisher). The HPLC mobile phase consisted of methanol (50% v/v)/phosphate buffer (50 mM, pH 7.4) (50% v/v), at a flowrate of 1 mL/min and column oven temperature of 35 °C. The UV detection wavelength for metoprolol was set to 274 nm.

2.2.5. Data analysis/permeability calculations

The cumulative amount of permeated drug (dn) was plotted as a function of time (t) and the area (A), according to:

$$J = \frac{dn}{A \cdot dt} \tag{1}$$

The linear part of the slope corresponded to the steady-state flux (J). In some of the permeability studies, a lag-time was observed. For these experiments the lag time was excluded when the flux was determined, hence all calculations were made during the steady-state flux. The obtained flux values were then used to calculate the apparent permeability coefficient (P_{app}) using Eq. (2), where the flux, was divided by the initial concentration of permeated drug:

$$P_{app} = \frac{J}{C_0} \tag{2}$$

2.2.6. Statistical analysis

Significant changes in permeability were evaluated by a two-sided student's t-test and ANOVA Tukey Post-hoc test. $P \leq 0.05$ was considered as significantly different. Potential outlier values were evaluated using a Thomson Tau test.

3. Results and discussion

3.1. Functional stability of Permeapad® at different pH values between 7 and 10

Earlier investigations (Holm et al., 2013; Meng-Lund et al., 2014a, 2014b) into pH dependent buccal absorption of metoprolol have used pH values of up to 9.5 since the pKa value of metoprolol is 9.3. Before investigating the permeability of metoprolol, the functional stability of Permeapad® was established in the pH range used in (Holm et al., 2013). Permeapad® has previously shown to withstand pH values up to 9. However, the results reported showed a slight permeability decrease at pH 9 for hydrocortisone (HC) (di Cagno et al., 2015). These

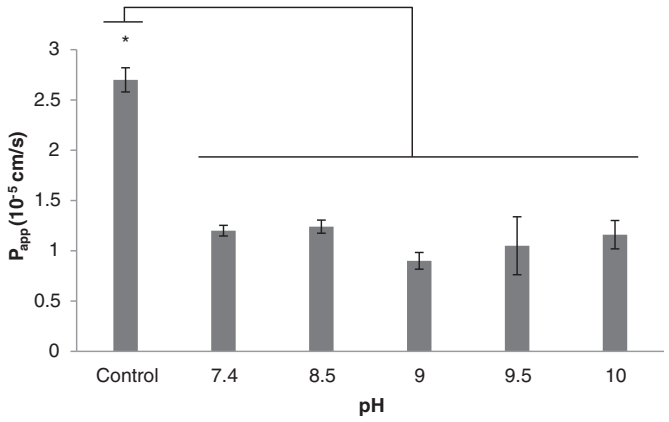

Fig. 1. Permeability of hydrocortisone from buffered saturated suspensions through the empty barrier support (Control) and through Permeapad® *vs.* the respective pH values. Data presented as mean ± SD (n = 3–4). *$P < 0.05$ compared to control.

data were based on single experiments, why the permeability experiments in the current study were carried out with more replicates using suspensions of HC in buffers of pH 8.5, 9.0, 9.5, and 10. Given the molecular structure of HC, no change in charge should occur over the evaluated pH range, why the compound should have a constant permeability at all the pH values, assuming that the barrier was not compromised by the buffer. Results in Fig. 1 show the apparent permeability coefficient for HC, in a control experiment using the empty barrier support at pH 7.4, and across the Permeapad® barrier at increasing pH values. The permeability of HC, at pH values as high as 10, was not affected and thus the integrity of Permeapad® was maintained. There appeared to be a significantly lower permeability at pH 9 as compared to pH 7.4 and 8.5 ($P < 0.05$), which likely is an artifact, because at pH 9.5 and pH 10, permeability was higher again and not different to the other values. Previous studies have shown that the integrity of Permeapad® was maintained at pH values as low as pH 1 (di Cagno et al., 2015). These data demonstrate the high functional stability of the barrier over a wide pH range, indicating its potential use for various pharmaceutically relevant applications. After establishing the functional stability of Permeapad® in the pH range used in the current study, the permeability of metoprolol at different pH values was studied.

3.2. Influence of buffer concentration on the P_{app} of metoprolol

The permeability of metoprolol at different pH values was determined using Permeapad® as a barrier using two different permeability setups. Metoprolol solutions were prepared as previously described in the *in vitro* and *ex vivo* studies carried out by Holm and coworkers (Holm et al., 2013). A phosphate buffer was used for the lowest pH value (7.4) and a borate buffer for the remaining pH values (8.5; 9.0 and 9.5). Holm et al. conducted their *in vitro* studies with a buffer concentration of 25 mM PBS/BBS, while a buffer concentration of 100 mM PBS/BBS was used for the *ex vivo* studies. This difference in buffer concentration was argued to be a reflection of sensitivity of the TR146 cells towards the higher buffer capacity (Holm et al., 2013). Results from the permeability studies are shown in Table 1.

Buffer concentration seems to affect P_{app}, with increased P_{app} when the lower buffer concentration of 25 mM was used. A *t*-test showed no significant difference ($P < 0.05$) for pH 8.5 and 9.5 between the two buffer concentrations, probably due to high standard deviations. In contrast, significant differences were noted for the two lower pH values. Since all buffers were adjusted to isotonic properties, the differences in P_{app} were not caused by osmotic differences. For both buffer concentrations, P_{app} values were plotted as a function of the pH values in accordance to Holm et al., 2013 (Fig. 2). Linear correlations ($R^2 = 0.97$ and $R^2 = 0.99$, respectively) were found (Fig. 2). This indicates that metoprolol is better permeable at higher pH values due to an increase of the non-ionized fraction (Holm et al., 2013; Meng-Lund et al., 2014a) notwithstanding the fact that based on pH theory by Henderson-Hasselbalch not a linear but rather the exponential part of the sigmoidal relationship to pH should be expected. However, as can be seen in Fig. 2, the slopes of the trend lines seem to be independent of buffer concentration – which indicates that the difference in P_{app} values was caused by the difference in buffer concentrations. Therefore the effect of the buffer concentrations should be kept in mind when comparing literature data, at least for studies conducted using the Permeapad® as the barrier.

The pH values in both the donor and acceptor chambers were measured before and after the permeation experiments. For the lower buffer concentration, a slight change in pH was measured in the donor chamber at the end of the experiment; 7.42 ± 0.01, 8.56 ± 0.01, 8.94 ± 0.01, and 9.36 ± 0.01. No significant pH changes were noted for the permeability studies carried out with 100 mM PBS/BBS buffers, which was expected due to very low drug concentrations (0.1 mM) compared to buffer capacity. These results demonstrated that the Permeapad® barrier was able to maintain a pH difference between the acceptor and donor chambers within reasonable variations for enough time to conduct studies investigating influence of pH on absorption. Results also show that Permeapad® can easily withstand the high buffer concentration, which is a clear advantage as compared to the TR146 cells.

3.3. pH dependent transport across Permeapad® and support sheet

The permeation of metoprolol across Permeapad® and the empty support sheet, respectively, has been studied at all four pH values. The results depicted in Fig. 3 show that the permeation of metoprolol across the support sheet is pH independent ($P > 0.05$); the transport mechanism is supposed to be simple diffusion. However, the same experiments carried out on Permeapad® show a clear increase of permeation with increasing pH. This means that the lipid layer in Permeapad® distinguishes between ionized (protonated) and non-ionized (free base) forms of the molecules. Permeapad® is in this respect not a sheer diffusion barrier but biomimetic. It is interesting to note that at higher pH values (pH 9 and 9.5) the lipid layer can increase the overall transport rate as compared to the sheer hydrophilic support sheet, probably due to the lipophilicity of the non-ionized drug (log *P* approx. 2) and the persistent pH gradient to the acceptor chamber (pH 7.4) mimicking *in vivo* conditions.

Table 1
The apparent permeability of metoprolol and the amount of permeated drug across Permeapad® at different pH values using two different buffer concentrations; 25 mM buffer and 100 mM buffer. Data presented as mean ± SD (n = 3–6). $P < 0.05$ for pH values 7.4 and 9.

	Apparent permeability (P_{app}) 10^{-5} cm/s		Amount of permeated drug, μmol/cm^2	
Buffer concentration	25 mM	100 mM	25 mM	100 mM
pH 7.4	2.36 ± 0.28	1.45 ± 0.08	0.0342 ± 0.004	0.0215 ± 0.001
pH 8.5	3.28 ± 0.95	2.81 ± 0.19	0.0503 ± 0.013	0.0424 ± 0.002
pH 9.0	4.26 ± 0.27	3.21 ± 0.29	0.0618 ± 0.021	0.0444 ± 0.004
pH 9.5	4.34 ± 0.49	3.63 ± 0.49	0.0631 ± 0.006	0.0574 ± 0.004

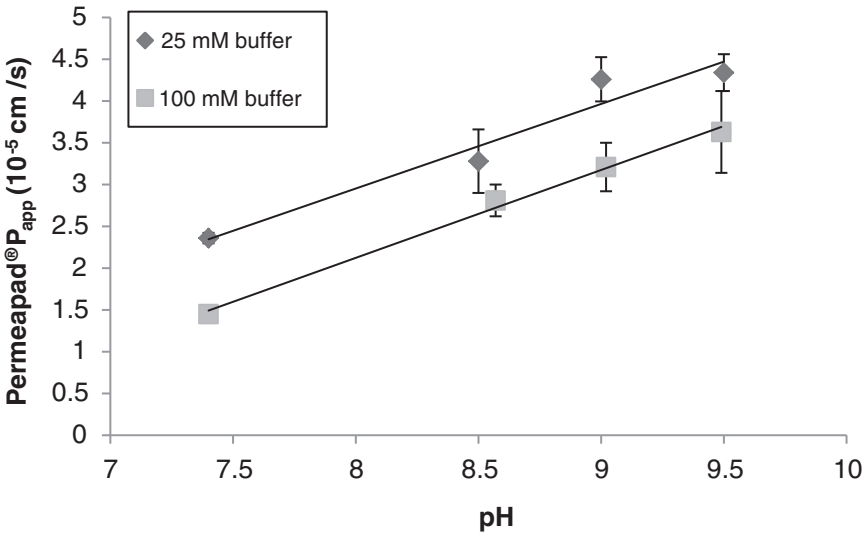

Fig. 2. Apparent permeability coefficients (P_{app}) of metoprolol through Permeapad® as a function of the pH values in 25 mM buffer and 100 mM buffer. Pearson correlation value obtained was 0.97 and 0.99, respectively. Data presented as mean ± SD (n = 3–6).

It is assumed that the lipid layer in Permeapad® forms bilayer structures (liposomes) upon contact with aqueous media, mimicking cell membranes; the packing of the liposomes may represent the epithelial tissue. The hydrophilic support sheet may mimic a mucus layer.

Based on the assumption that only the non-ionized fraction of the drug permeates through phospholipid bilayers, *in vivo*, across Permeapad®, cell layers, and tissues, linear correlations between P_{app} (calculated using the total drug concentration) and the non-ionized fraction of the drug at the respective pH value should be expected. In Fig. 4 P_{app} is plotted *vs.* the respective fraction of non-ionized metoprolol, comparing the values from the present study (data from Table 1) with those from Holm et al. for cell cultures and *ex vivo* tissues (Holm et al., 2013). Fig. 4 clearly shows that the relationship between the P_{app} and non-ionized fraction of metoprolol is not linear as expected but in all cases the slope decreases with increasing concentration of the non-ionized drug to reach a plateau, above which saturation of the transport capacity is observed. It is interesting to note that Permeapad® shows permeability limitations in the same range as the other methods.

3.4. Drug recovery

Drug recovery for Permeapad® was calculated from the concentration in the donor chamber after the permeation experiment and the cumulated amount of drug in the acceptor chamber. The drug recoveries obtained were 97 ± 3.2%, 86.2 ± 15.6%, 95.1 ± 11.4% and 93.8 ± 10.2% for the increasing pH values, respectively. For the empty support sheet, the values were 108.3 ± 1.7%, 104.6 ± 2.9, 95 ± 5.1% and 95.2 ± 2.8%, for the respective pH values. These data show that drug recovery is in general high and close to 100%, considering accumulation of experimental errors. A tendency towards lower drug recoveries in Permeapad® as compared to the support sheet, indicate that a small fraction of metoprolol may adhere to the lipid layer. Permeapad® has earlier been studied for drug recovery for a variety of different drugs with different log *P* values (up to >4), and high drug recoveries (90–100%) were reported (di Cagno et al., 2015; Bibi et al., 2015). In this respect Permeapad® has an advantage as compared to the PAMPA model in which lipophilic drugs tend to accumulate in the lipidic phase thereby reducing the concentration in the acceptor phase (Avdeef, 2005). Another artificial lipid barrier widely used in permeability testing is the Phospholipid vesicle based permeation assay (PVPA), which also suffers from low drug recoveries (below 50%) for lipophilic drugs (Naderkhani et al., 2015). Recently, modified versions of both PVPA and PAMPA assays mimicking the skin have been introduced (Sinkó et al., 2012; Engesland et al., 2013). Permeation experiments on PVPA using mucoadhesive liposomes have been carried out, and the authors claim that the PVPA barrier has the potential to serve as a general model mimicking different absorption barriers (Naderkhani et

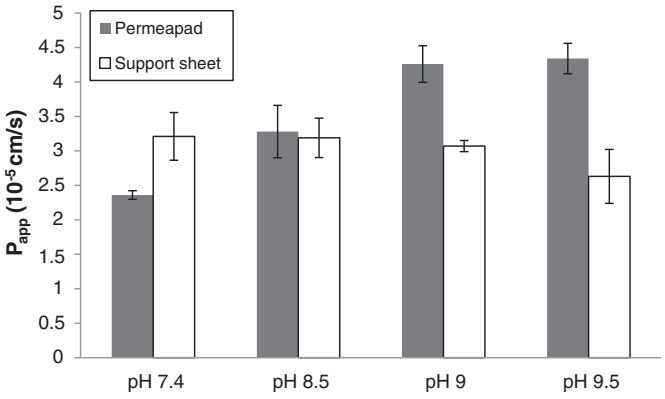

Fig. 3. Apparent permeability coefficient (P_{app}) of metoprolol at different pH values through Permeapad® and through the empty barrier support (25 mM buffer). Data presented as mean ± SD (n = 3–6).

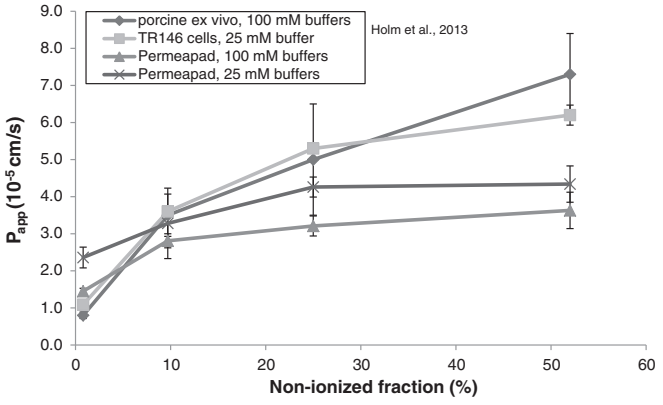

Fig. 4. Apparent permeability coefficients (P_{app}) of metoprolol through Permeapad® *vs* non-ionized fraction of the drug in the respective 25 mM and 100 mM buffers. Data from Holm et al., 2013 in the same buffers for porcine tissue and TR146 cells are plotted for comparison.

al., 2014). However, none of the models has yet been modified for mimicking buccal mucosa.

3.5. In vitro permeability studies in TR146 cell culture and porcine buccal mucosa compared to Permeapad® permeability studies

Direct comparison of P_{app} values for Permeapad® with published data for the TR146 cells and porcine buccal mucosa (Holm et al., 2013) showed excellent correlations, with Pearson correlation coefficients of 0.98 and 0.97, respectively (Fig. 5A and B). However, in both cases, Permeapad® showed a more narrow range of P_{app} values as compared to both the TR146 cells and the porcine buccal mucosa, *i.e.* the relationship was not 1:1. On the other hand, experiments with Permeapad® were better reproducible as indicated by their lower SD, which yields significant differences. The results therefore indicate that Permeapad® may be used as an alternative and less laborious method to predict the buccal absorption of metoprolol instead of the TR146 cells and porcine buccal mucosa.

The TR146 cell culture originates from human buccal carcinoma that has been shown to mimic the buccal mucosa well (Mørck Nielsen and Rømer Rassing, 2000). As is the case for many cell models the TR146 cells require a cultivation period of 27–30 days before this specific cell line can be used (Holm et al., 2013; Mørck Nielsen and Rømer Rassing, 2000), why it is obviously more time consuming to use as compared to Permeapad®. The same is true for the *ex vivo* assay, for which tissues need to be received and prepared. The results indicate - upon confirmation with more drug substances- that Permeapad® may be useful as a surrogate for cell and tissue studies. This is particularly interesting as Meng-Lund et al. (2014a, 2014b), through morphological studies of the buccal tissue from porcine and humans have demonstrated that

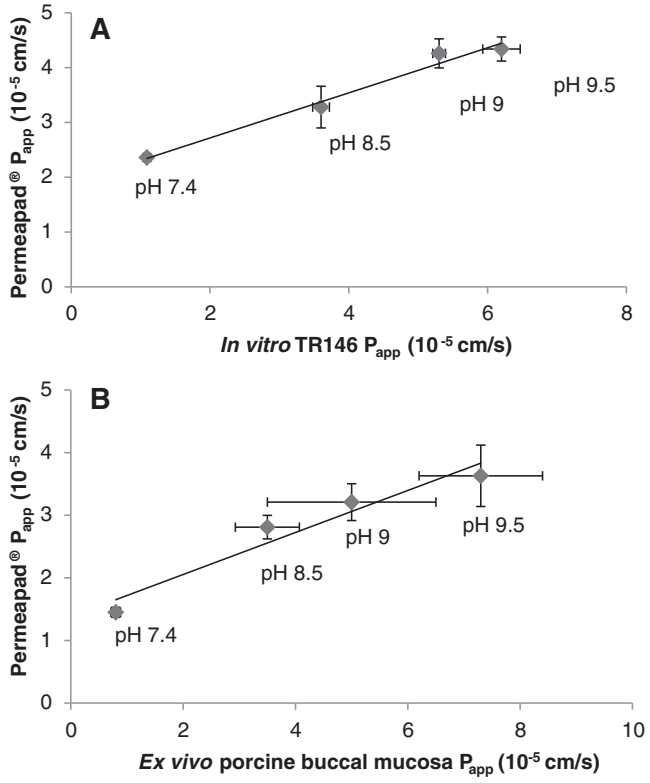

Fig. 5. Correlation between apparent permeability coefficient (P_{app}) of metoprolol through Permeapad® and (A) *in vitro* TR146 cells from Holm et al. (2013); Pearson correlation value obtained: 0.98; data presented as mean ± SD (n = 6) for Permeapad® and (n = 4) for TR146, and (B) *ex vivo* buccal mucosa (Holm et al., 2013); Pearson correlation value obtained: 0.97. Data presented as mean ± SD (n = 3) for Permeapad and (n = 4) for *ex vivo* buccal mucosa.

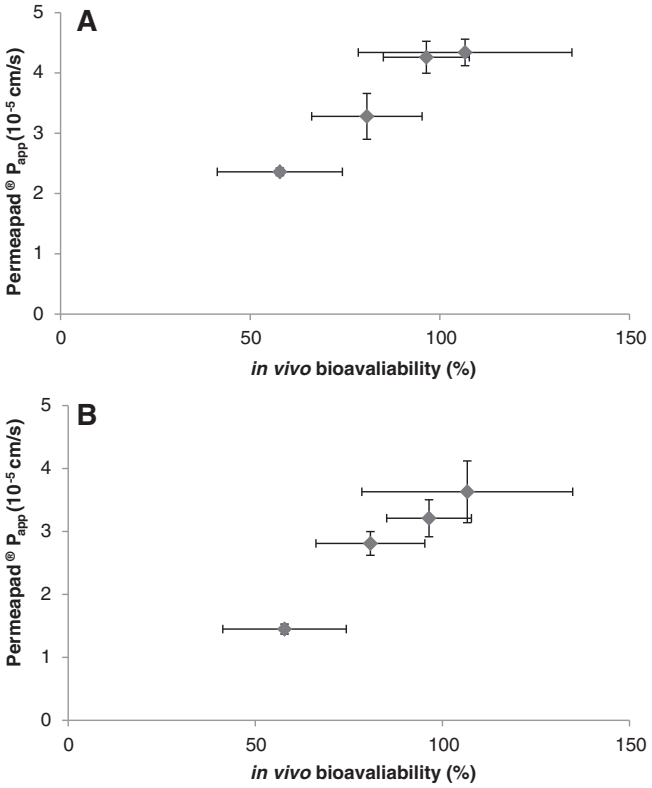

Fig. 6. Correlation between *in vivo* absolute bioavailability of metoprolol obtained from mini-pigs administered buccally with metoprolol gels (Holm et al., 2013) and (A) apparent permeability coefficient (P_{app}) of metoprolol through Permeapad® in 25 mM buffer. Pearson correlation value obtained: 0.98. Data presented as mean ± SD (n = 6) for Permeapad® and (n = 4) for mini pigs, and (B) P_{app} of metoprolol through Permeapad® with 100 mM buffer. Pearson correlation value obtained: 0.98. Data presented as mean ± SD (n = 3) for Permeapad® and (n = 4) for mini pigs.

the porcine model may be used as a replacement for human tissue samples (Meng-Lund et al., 2014b).

3.6. In vivo permeability studies with metoprolol compared to Permeapad® permeability studies

In order to determine whether Permeapad® can be used to make *in vitro–in vivo* correlations (*IVIVC*), the data generated in the permeability setup with Permeapad® barrier was compared to reported *in vivo* data from anesthetized Göttingen minipigs (Holm et al., 2013). The P_{app} values of metoprolol obtained for both buffer concentrations were compared to the absolute bioavailability from the Göttingen minipigs as shown in Fig. 6A and B. A Pearson correlation value of 0.98 was obtained for both buffer concentrations, indicating a very good correlation, between the P_{app} from the Permeapad® permeability studies and fraction absorbed (FA) in the *in vivo* studies.

Studies carried out by Holm et al., 2013 (Holm et al., 2013), comparing the absolute bioavailability from *in vivo* studies and *ex vivo* studies also showed a good correlation, indicating that *ex vivo* studies is a very suitable method to determine buccal permeability of drugs. However, Permeapad® is a faster method for early permeability screening, and the present study indicates that it can be used to determine permeability of drugs for buccal administration, although the method still needs to be validated for more drug substances in addition to metoprolol.

4. Conclusion

Permeability of the model drug metoprolol was determined across Permeapad® and compared to literature data for permeation across TR146 cell layers, porcine buccal mucosa *ex vivo* and in Göttingen

minipigs. The results showed a good correlation between Permeapad® and the respective *in vitro* studies, which indicated that Permeapad® appears useful as a predictive assay for pH dependent permeability for this basic drug substance. Permeapad® is suggested as a preliminary permeability tool for buccal absorption of metoprolol. An excellent *IVIVC* ($R^2 = 0.98$) was obtained when comparing P_{app} to the absolute bioavailability of metoprolol administered buccally in the form of a gel to mini-pigs, indicating that in the case of metoprolol Permeapad® can be used to mimic buccal mucosa as a faster and less laborious method as compared to any of the other mentioned methods.

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