

Evaluating the influence of UWL and its composition on permeability profiling of two model drugs by using the PermeaPad[®] Plate and 2mag MIXdrive 96 MTP magnetic stirrer

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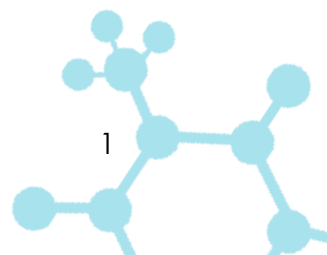
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Introduction

New chemical entities (NCE) synthesized in pharmaceutical laboratories across the world must have desirable physicochemical characteristics like solubility, membrane/aqueous partition coefficient etc. (1) before they are investigated in *in vitro* permeability assays. These assays, as their name imply, will determine the apparent permeability of NCE and decide their further destiny as possible active pharmaceutical ingredients. Apparent permeability as molecule property is a composite of the membrane effect and the unstirred water layer (UWL) adjacent to both sides of the barrier. UWL, as stagnant layer, acts as diffusion barrier and rate-limiting factor for rapidly permeating, highly lipophilic drugs. For hydrophilic drugs, the velocity of permeation process will be determined by the permeability through the membrane since the membrane (not the UWL) is the slow step in permeation for such drugs. Many plate-based *in vitro* assays report permeability in water instead the permeability of the membrane itself (2), leading to underestimation of the mitigating effect of UWL on permeability, which leads to poor correlation between experimental values and fraction absorbed (i.e.

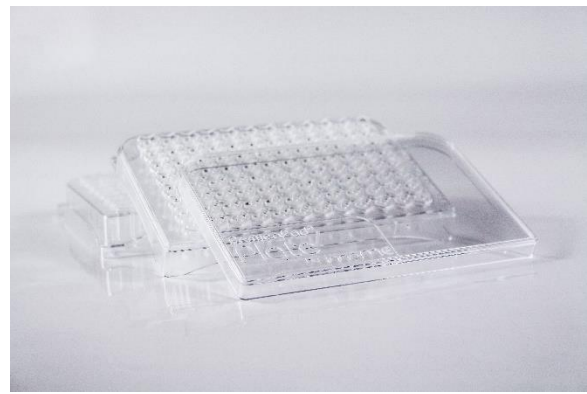
bioavailability) in humans. Although considerable effort has been put in developing new permeability models with various types of biomimetic barriers, the influence of the unstirred water layer (UWL) remains poorly studied in such assays. UWL is formed in close proximity of any barrier (biologic or artificial) due to insufficient/inefficient stirring, which leads to a concentration gradient between the bulk solution and the barrier. It is believed that UWL acts as an additional permeability barrier by reducing the net flux and, as a consequence, the apparent permeability coefficient (P_{app}) of drugs through a barrier.

The aim of this work was to examine the contribution of UWL as an additional barrier on permeability of two model drugs – caffeine (CAF) and hydrocortisone (HC) using PermeaPad[®] 96-well plate under different experimental conditions. CAF and HC are a water-soluble /highly permeable and a poorly soluble and medium/low permeable compound, respectively. Experiments were conducted with different rates and types of agitation/stirring pH values and donor solutions with various API concentrations





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Experimental set-up

Solutions

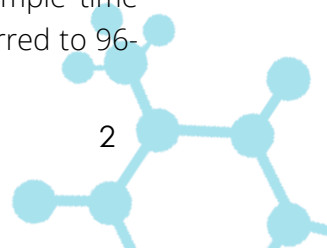
Phosphate-buffered saline (PBS) 73 mM pH 7.4 was prepared from two solutions: 2.2% (w/v) sodium dihydrogen phosphate dihydrate with 0.9% (w/v) di-sodium hydrogen phosphate dihydrate mixed in a ratio of 1:4. The pH was adjusted to 7.4 (± 0.05) by adding sodium hydroxide pellets (measured by pH meter Mettler Toledo seven compact, USA). Osmolality was adjusted with sodium chloride to the value 285-300 mOsmol/kg. Test compounds – caffeine (CAF), calcein (CAL) and hydrocortisone (HC) – were dissolved in PBS in various concentrations 0.5 – 5 mM (depending on the solubility of each drug or on the intended purpose of experiment) by use of sonication bath Transsonic 460/H (Elma Schmidbauer, GmbH, DE).

Solutions were diluted to the adequate concentration and concentration of the drug was then determined spectrophotometrically. All samples were transferred to 96-well UV-transparent plates (Costar®, Corning Incorporated, USA) and concentration was determined by measuring absorbance at the end of experiment. Experiments were performed in three or six replicates ($n=3 - 6$) (6 replicates for the initial

experiments as a mean to evaluate method variability). Absorbance of all samples was measured by Spectramax 190 microplate reader at the appropriate wavelength for each drug where the substance has its strongest photon absorption in PBS according to its wavelength scan – 273 nm for caffeine, 248 nm for hydrocortisone and 491 nm for calcein. Some of the samples had to be diluted with PBS to obtain a range of concentrations that correspond to the concentrations of the standard curves where Lambert-Beer law's linearity was still maintained.

Membrane integrity

For validation of the membrane's integrity, 200 μ l of 2 mM calcein solution (MW=622.6 g/mol) were used in the donor compartment and 400 μ l PBS as acceptor solution. Permeability experiment was conducted in orbital shaker at 200 rpm and 25 °C, direction top to the bottom (T-B) for a total of 24 hours. There were 6 parallels and 8 sample points (every 30 minutes). The sample volume (120 μ l) was replaced with an equal amount of fresh PBS at each sample time point. Samples were then transferred to 96-





well UV-transparent plates and absorbance was measured by Spectramax 190 microplate reader at $\lambda=491$ nm. Stock solution for the calibration curve had concentration of 1 mM, range of concentrations for calibration curve was 0.0125 – 0.025 mM and R^2 was higher than 0.99.

Diffusion experiment

The top to the bottom (T-B) approach was chosen in this project. For T-B orientation, 200 μ l of a 2 mM caffeine solution were used in the top (donor) compartment and 400 μ l PBS – in the bottom (acceptor) compartment. Samplings were performed at 15, 30, 45, 60, 90, 120, 150, 180 and 210 minutes after start of experiment. The multi-well PermeaPad® Plate was placed in an orbital shaker or magnetic stirrer (MIXdrive

96 MTP magnetic stirrer, 2mag, Germany) at 200 rpm and 25 °C for 3.5 hours (n=6). Absorbance was recorded by a Spectramax 190 microplate reader (Molecular devices Corporation, USA).

Results

In each experimental setup it is important to validate integrity of the membrane. The most used way is to measure permeability for a hydrophilic marker, in this work – calcein. Low permeability for calcein is an indication that calcein does not go through the lipid layer and that the layer is intact. Increasing of calcein's P_{app} would indicate a significant lost in barrier integrity. Flux of calcein $(2.79 \pm 1.14) \times 10^{-7}$ cm/s was approx. 50-fold lower than flux for caffeine 2 mM solution $(1.40 \pm 0.05) \times 10^{-5}$ cm/s. In Figure 1 the diffusion profiles of the different experiments are reported.

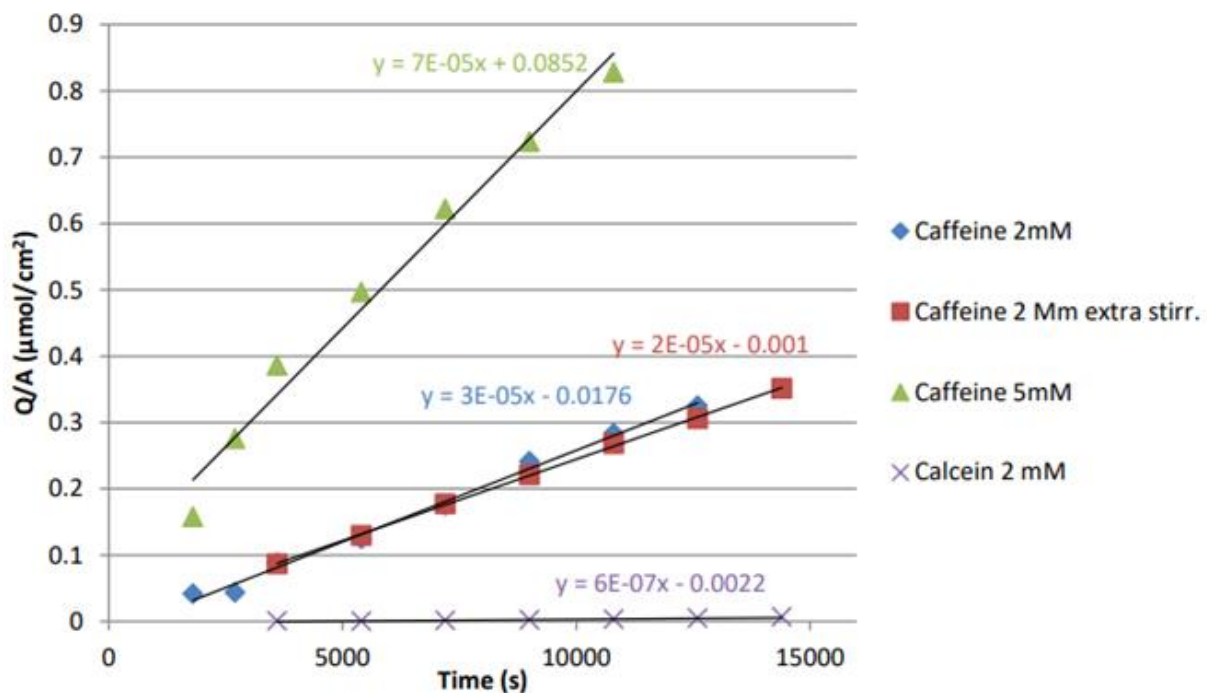
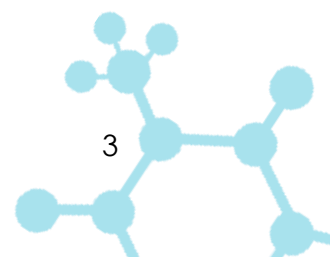


Figure 1: Cumulative mass that permeated through the membrane normalized to permeation area and plotted against time for caffeine and calcein at 200 rpm in orbital shake (3).





Permeability profiling – influence of stirring

It should be underlined that, P_{app} is a constant at given conditions (e.g. temperature, pH, stirring etc.). Alteration of conditions might affect P_{app} values. For instance, significant alteration of stirring conditions can enhance or reduce the absolute P_{app} values for a given drug, due to perturbation of UWL.

The impact of UWL is poorly studied in *in vivo* and *in vitro*. Stirring can have a significant

impact on UWL thickness and therefore on the net permeability measured. Use of small magnets moved by magnetic stirrer was, in general, more effective than agitation in orbital shaker as can be seen from figure 2&3. Hydrocortisone results show higher standard deviation values in comparison to caffeine which is expected due to its lower permeability compared to the permeability of caffeine. Obtained results for CAF for both agitation methods and three different magnet stirring rates are given in Figure 2 while results for HC are given in Figure 3.

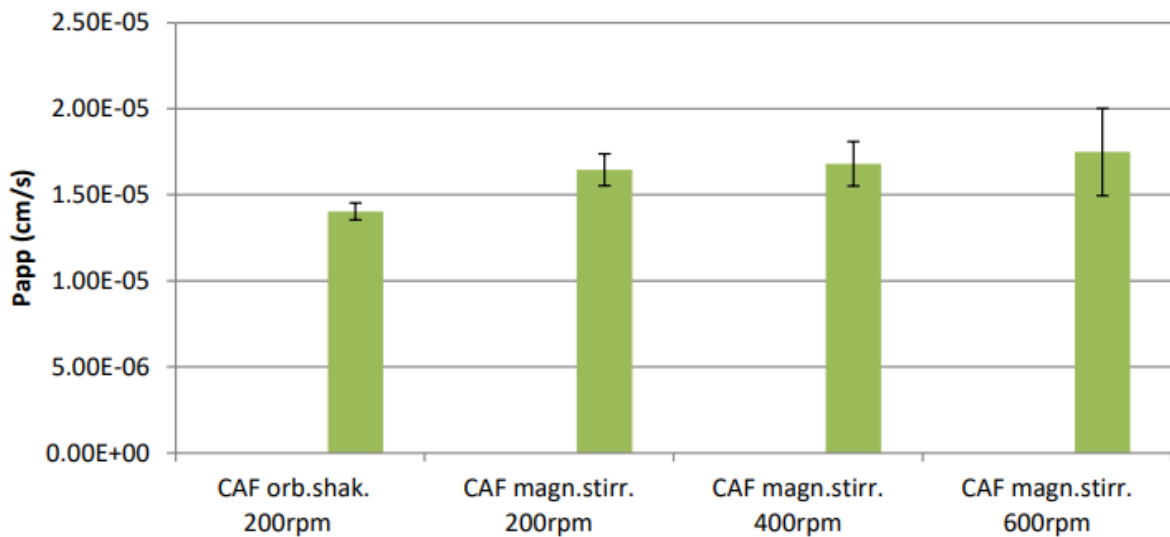


Figure 2: P_{app} with SD for 2 mM CAF at different stirring rates in orbital shaker (orb.shak.); (n=6) and magnetic stirring (magn.stirr.); (n=3) (3)

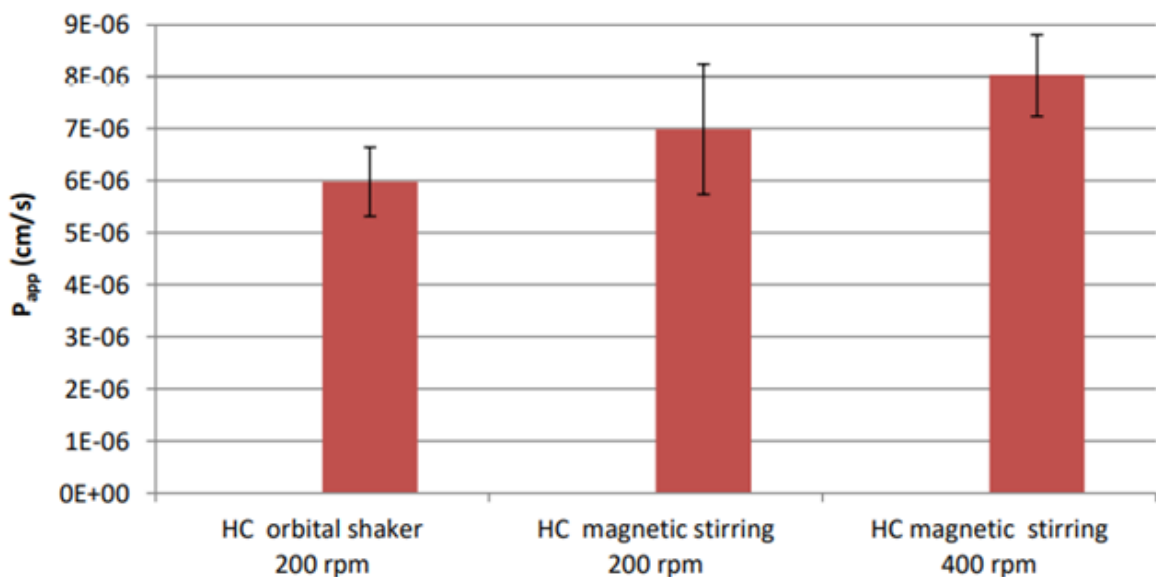
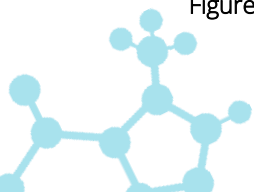


Figure 3: P_{app} with SD (n=3) for 0.5 mM HC for different types of agitation (3)





Conclusion

The unstirred water layer can be considered as a significant additive to permeation in *in vitro* experiments and its effect should be carefully analyzed in order to acquire permeability values that correlate better with the fraction absorbed in humans. The use of small magnets moved by magnetic stirrer from 2mag was, in general, more effective than agitation in orbital shaker. Highly permeable drugs, such as caffeine are not significantly affected by the presence of UWL. In literature it is possible to find that that UWL has an impact on permeability of drugs with high transport rate which is opposite to the effect of UWL on caffeine. It is an argument for experimental determination of the permeability coefficients values for each drug and further, that methods for lowering the UWL thickness should be carefully considered for each drug separately. On the other hand, medium/low permeable HC resulted highly affected by UWL and its thickness/composition.

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Supplement

Passive diffusion is generally described by Fick's first law of diffusion:

- constant concentration gradient ($c_d - c_a = \Delta c$; $dx=h$)

$$j = D * \frac{(c_d - c_a)}{h} \quad \text{Obtained by sink conditions}$$

- $c_d \gg c_a$ ($C_a \approx 0$ but not zero)

$$j = D * \frac{c_d}{h} \quad \text{Obtained by choosing proper donor drug concentration}$$

- D constant all over the system

$$P_{app} = \frac{j}{c_d} \quad \text{Papp is known as "apparent permeability coefficient" and it is a velocity (e.g cm/sec)}$$

- j = Flux
- D = Diffusion constant
- c_d = Donor drug concentration
- c_a = Acceptor drug concentration
- h = time
- P_{app} = Apparent permeability coefficient