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‘Stirred not Shaken!’ Comparing Agitation Methods for Permeability Studies Using a Novel Type of 96-Well Sandwich-Plates

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ABSTRACT

In order to achieve a high sample throughput, permeation experiments are often carried out using 96-well sandwich plates. Even though agitation is regarded as important, permeation studies in 96-well format are often carried out without agitation since orbital shaking, the most common agitation method for 96-well plates, has been reported to create difficulties (e.g., well-to-well cross-talk), and high cost and low availability limits the use of other agitation techniques (e.g., magnetic stirring). This study investigates how orbital shaking and magnetic stirring affect the apparent permeability of model compounds with different water-solubilities (methylene blue, carbamazepine, and albendazole) using a novel 96-well sandwich plate comprising a cellulose-hydrate membrane (PermeaPlain® plate). Orbital shaking was found less efficient than magnetic stirring in terms of homogeneously distributing a small volume of dye within the donor compartment. Furthermore, in terms of achieving maximum trans-barrier flux, magnetic stirring was found a more effective agitation method than orbital shaking. Obviously, with orbital shaking the medium in the bottom compartment of the sandwich plates never was mixed in-phase. The impact of insufficient mixing on permeation was found strongest with the most lipophilic compound, which correlates with literature reports that the contribution of the unstirred water layer towards the overall resistance of the barrier is most expressed in case of lipophilic drugs. Finally, it was tested how different liquid volumes in the bottom compartment of the plates affect the well-to-well cross-talk during permeation experiments under orbital shaking. This study revealed that 250–300 μL should be used in the bottom compartment of the sandwich plates to reduce well-to-well cross-talk when using orbital shaking for agitation.

Introduction

Both, regulators and scientists in drug development widely use in vitro permeation tools, such as Caco-2, PAMPA, PVPA or PermeaPad® for biopharmaceutical characterization and classification of drug compounds.[1,2] In drug discovery, the permeability of large compound libraries preferably is screened using 96-well plate-based approaches in high throughput.[3] More recently, it has also been suggested to use these in vitro tools in formulation-development: Jacobsen et al. introduced combined dissolution/permeation experiments in 96-well format to evaluate the performance of enabling formulations of poorly soluble drugs with very promising results in terms of predictability of bioavailability.[4,5]. They either used a 96-well sandwich plate comprising a cellulose-hydrate membrane[4] (i.e. PermeaPlain® Plate) or the phospholipid-based PermeaPad® membrane[5] (i.e. PermeaPad® Plate). Such combined dissolution/permeation testing may reveal the interplay between permeation and supersaturation, as well as allow for discrimination between apparently dissolved and molecularly dissolved drug, an information that traditional dissolution tests typically fail to provide.[6–9]. Here, we are using the terms apparently dissolved and molecularly dissolved. Apparently dissolved describes all dissolved species including single solute molecules surrounded by a solvation shell (molecularly dissolved) and solubilized molecules incorporated within a micelle or associated with a complex forming agent such as cyclodextrin.

Agitation of liquids has a significant influence on the kinetics of dissolution tests and permeation studies. In dissolution testing, agitation

Abbreviations: BCS, Biopharmaceutical classification system; Caco-2, colon carcinoma cell line; EMA, European Medicines Agency; FaSSIF, Fasted state simulated intestinal fluid; PAMPA, Parallel artificial membrane permeability assay; P_{app}, Apparent permeability; PVPA, Phospholipid Vesicle Permeation Assay; TPGS, D-α-Tocopherol polyethylene-glycol 1000 succinate; UHPLC, Ultra high performance liquid chromatography; UV/VIS, Ultraviolet / visible spectroscopy; UWL, Unstirred water layer.

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is important to reduce the diffusion layer thickness on the tablet surface, to de-agglomerate particles and to mix tablet fragments. Agitation also influences the mass transfer from the solid to the liquid.[10,11] In permeation studies, agitation of the donor compartments often enhances the permeation rate of poorly soluble compounds.[12] This enhancement often is regarded to be caused by a reduction of the thickness of the unstirred water layer (UWL), which is a diffusion barrier of unstirred aqueous buffer adherent to the permeation barrier and which the compound needs to overcome. Studies indicate that the thickness of the UWL is depending on, e.g., the nature of the barrier and the geometry of the permeation set-up.[12–15] Agitation of the donor and acceptor compartments are observed to increase the permeation rate of poorly soluble compounds, which is thought to be due to a decrease of the UWL thickness. Clearly, effective agitation of 96-well permeation devices is regarded a pre-requisite to obtain in vivo relevant permeability predictions.[12]

Paddle stirring is the mixing method of choice in dissolution testing.[16] Despite the substantially smaller scale, today, paddle stirrers for 96-well plates are commercially available (ULVAC Technologies Inc.). However, such paddle stirrers are not an option for permeation studies in 96-well plates since it is practically impossible to reach the bottom compartment, which typically is used as the donor compartment. Magnetic stirring is an alternative approach suited for the bottom compartment. With respect to magnetic stirring, a drawback was observed: stir bars in neighboring wells tended to stick together at the wall between wells due to the small distance between wells. This could be avoided by using alternate wells only, leading to ineffective use of plates, however, because half of the wells needed to be kept empty (see supplementary material 1). For these reasons, agitation of 96-well plates during permeation studies is most commonly conducted using orbital shakers (i.e. rotating platforms), a cheaper and readily available equipment as compared to 96-well paddle stirrers and magnetic stirrers. The hydrodynamics in 96-well plates, which are mixed by rotating platforms depend on several factors such as well geometry and rotation speed.[17] Despite the difficult hydrodynamics, rotating platforms have been shown to reduce UWL thickness in Transwell® systems employed within Caco-2 assays.[14,17] In contrast, other reports indicate, that shaking using rotating platforms is insufficient in 96-well PAMPA plates.[18] Obviously, choosing a suitable agitation method for permeation experiments in 96-well plates is not a trivial task. This may also be the reason, why permeation experiments often are conducted without any agitation.

This study systematically compares two agitation methods for mixing of liquids in 96-well plates by examining the effects of orbital shaking and magnetic stirring on the apparent permeability of three model compounds methylene blue, carbamazepine and albendazole in a novel 96-well sandwich plate, which has a more complicated geometry than other 96-well plates (i.e. the geometry is similar to the PermeaPad® plate, which is described in detail by Jacobsen et al.[19]). The three model compounds were chosen to cover different hydrophilicities/lipophilicities. Methylene blue[20] is hydrophilic, carbamazepine[21] is of intermediate hydrophilicity / lipophilicity and albendazole[22] is rather lipophilic. Different stirring and shaking speeds and a surfactant were employed in an attempt to understand the mechanisms that affect the mixing of liquids in a model 96-well plate and its potential effect on the unstirred water layer. Furthermore, well-to-well cross-talk while shaking the plates, which is a commonly observed phenomenon while mixing microplates,[23] is systematically evaluated.

Materials and Methods

Materials

Albendazole (purity 98 %) was purchased from abcr GmbH, (Karlsruhe, Germany). Carbamazepine, dipyridamole (≥ 98 %), sodium phosphate dibasic dihydrate (purity ≥ 98 %) and sodium phosphate monobasic monohydrate (purity ≥ 98 %) were purchased from Sigma-Aldrich (St. Louis, USA). Methylene blue (purity 95 %) was purchased from Honeywell Riedel-de-Haën™ (Hannover, Germany). D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) was a kind gift of Gustav Parmentier GmbH (Frankfurt, Germany). Trifluoroacetic acid (purity 100 %) and methanol (purity 99.9 %) for the HPLC were purchased from VWR (Søborg, Denmark). All other compounds were of analytical grade.

Visual Observation of Methylene Blue Mixing With Regard to Agitation in a 96-Well Plate

Experiments to visually evaluate the mixing of liquids were carried out using the sandwich plate (PermeaPlain® plate, LOT: 20200610, InnoME GmbH, Espelkamp, Germany). This plate has the same geometry as the PermeaPad® plate, as described in Jacobsen et al. 2020[19], yet a different barrier composition. To visually evaluate the progress of mixing of two aqueous liquids, the bottom wells were pre-filled with 300 μL of water, to which 5 μL of a concentrated methylene blue solution were added. The mixing of undissolved particles was evaluated by adding 300 μL water to wells that already contained ~1 mg solid dipyridamole, a poorly soluble yellow colored drug compound. Liquids were added with the top plate already attached using a fine pipette through a hole on the top plate that enables sampling from the bottom plate. Then the plates were immediately agitated by magnetic stirring, orbital shaking, or left on the lab bench with no agitation. Orbital shaking was carried out at 300 rpm on a Thermo-Shaker PHMP-4 from Grant Bio (Cambridge, United Kingdom) (i.e. shaking in orbitals of 2 mm radius) and magnetic stirring was carried out at 100 rpm on a 96-well plate magnetic stirrer (2mag AG, Munich, Germany) using 5 × 2 mm stir bars. To evaluate the mixing, videos were captured throughout the process with a Huawei P20 Pro smartphone, and representative frames of those videos were selected and are presented in this work.

Preparation of Media

A 30 mM phosphate buffer was prepared by dissolving 10.7 mM sodium phosphate dibasic dihydrate and 19.3 mM sodium phosphate monobasic monohydrate in purified water (Millipak® Express, filtered through a 0.22 μm filter). Thereafter, the pH value was adjusted to 6.5 with hydrochloric acid (827 pH lab from Metrohm, Herisau, Switzerland). For permeability experiments, four different donor media were prepared: 1) A methylene blue solution was prepared by dispersing 2 mg/mL methylene blue in 30 mM phosphate buffer followed by approximately five min of sonication. Prior to the permeation experiment, the methylene blue solution was incubated for 20 min in the heating cabinet at 37 °C under stirring. 2) A carbamazepine solution was prepared by dispersing 100 μg/mL carbamazepine in 30 mM phosphate buffer followed by approximately five min of sonication. To fully dissolve the carbamazepine, the dispersion was then stirred at room temperature overnight. Prior to the permeation experiments, the carbamazepine solution was incubated for 20 min in the heating cabinet at 37 °C under stirring. 3) A suspension of albendazole was prepared by dispersing 100 μg/mL albendazole in 30 mM phosphate buffer the day before permeation experiments. The suspension was stirred overnight at room temperature. One hour before the permeation experiments, the suspension was incubated at 37 °C in the heating cabinet under stirring. 4) A albendazole suspension containing 1 % TPGS was prepared by the same procedure with one exception: 1 % TPGS was completely dissolved in the 30 mM phosphate buffer before albendazole was added.

Permeation Experiments

The permeation experiments were carried out using the novel sandwich plate. The bottom compartments (i.e. donor) were filled

with 400 μL of either the 2 mg/mL methylene blue solution, the 100 μg/mL carbamazepine solution, or one of the 100 μg/mL albendazole suspensions. In order to avoid cross-contamination through well-to-well cross-talk, no adjacent donor wells were filled with different donor media. The top compartments (i.e. acceptor) were filled with 200 μL 30 mM phosphate buffer. After sealing with VWR® precut pierceable vinyl films for 96 well plates from (VWR, USA), the plates were incubated in one of the following four settings: 1) a 96-well plate orbital shaker with temperature control (Thermo-Shaker PHMP-4) at 0, 250, 300 or 600 rpm, 2) a 96-well plate magnetic stirrer (MIXdrive 96 MTP) at 0, 100 or 600 rpm placed inside a heating cabinet (model 115, Binder, Tuttlingen, Germany), 3) without agitation but placed inside the heating cabinet and 4) without agitation and without temperature control (i.e. placed on the lab bench). In set-ups 1-3, the temperature was set to 37°C, while set-up 4 was at room temperature. Samples of 50 μL were drawn from acceptor wells in studies with carbamazepine and methylene blue solutions after 60, 90, 120, 150, and 180 min and for the studies with albendazole suspensions after 180, 240, 300, and 360 min. After sampling, the acceptor wells were immediately replenished with 50 μL fresh buffer so the next sample could be drawn from the same well. All samples were quantified according to paragraph 2.6, and the apparent permeabilities (P_{app}) for methylene blue and carbamazepine experiments or flux for albendazole experiments were calculated according to Section 2.7.

Investigation of Well-to-Well Cross-Talk

Three 96-well sandwich plates had all bottom wells filled in a chessboard pattern with a concentrated methylene blue solution and water. All wells on a plate were filled with the same volume, but different filling volumes were tested on the three plates, namely 250, 300, or 400 μL. After filling the bottom-plates, the top plates were added on to the bottom plates, and the assembled plates were placed into the Thermo-Shaker PHMP-4. The whole process, including filling of the wells and placing the plates in the orbital shaker, was carried out with extra awareness of careful handling to avoid mixing of methylene blue solution and water prior to the shaking. The plates were then shaken for 3 h at 300 rpm. After the incubation, the plates were visually inspected for well-to-well cross-talk indicated by the blue color of methylene blue.

Quantification

Methylene blue and carbamazepine were quantified by Ultraviolet-Visible (UV/VIS) spectroscopy at a detection wavelength of 291 and 300 nm, respectively, using a BMG FLUOstar® Omega microplate reader (BMG LABTECH,Germany).

Albendazole was quantified by ultra-high performance liquid chromatography with ultraviolet detection (UHPLC-UV) on an UltiMate 3000 UHPLC system (Thermo Fisher Scientific™, Waltham, Massachusetts, USA) connected to a reversed-phase C18 LC column (Kinetex®,1,7 μm, 150 × 2.1 mm, Phenomenex®). UV detection was performed at 294 nm using a diode array detector. The eluent consisted of 45% of methanol and 55% of 0.1% trifluoroacetic acid in purified water. The elution was isocratic with a flow of 0.25 mL/min and the column oven was set to 40 °C. The run time was 5 min, where albendazole eluted at 3 min. All quantifications have been conducted in parallel with freshly prepared calibration curves.

Calculations and Statistics

The flux (J) of the model compounds was calculated by normalizing the slope (dQ/dt) of the linear parts of the permeation curves (i.e. the cumulative amount of compound plotted against time) with the surface area (A) of the membranes (A = 0.15 cm^2).

$$J = \frac{dQ}{dt \cdot A}$$

To calculate the apparent permeability (P_{app}), the flux was normalized by the concentration (C_0) of the model compounds in the donor compartment.

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

Permeation data is presented as the mean of 5−10 replicates. Statistical analysis on the permeation data was carried out using GraphPad Prism 8.4.2 software. One factor ANOVA with Tukey's multiple comparisons test was utilized to identify significant differences among the means.

Results

Visual Observation of Donor-Well Mixing-Patterns

Fig. 1 depicts the progress of mixing patterns of a small volume of aqueous dye solution added to the donor compartment when using magnetic stirring or orbital shaking for agitation as compared to no agitation. In the case of magnetic stirring, the methylene blue solution was mixed almost immediately, and after 10 s the blue color was found homogeneously distributed all over the volume of the well. In contrast, when using orbital shaking, the added methylene blue solution was not mixed immediately with the water. As depicted in Fig. 1, after 30 sec of orbital shaking a much darker shade of blue is still observed on the well's left side, where the methylene blue had been added, as compared to the right side. Uniformity of color is first observed with orbital shaking after 2 min. When no agitation was applied, it was observed that even after 2 min, the methylene blue solution still was not well distributed within the volume of the well. In the next step, Dipyridamole, a yellow-colored drug compound with low water solubility, was used to investigate how suspended drug particles moved during agitation. When using magnetic stirring for agitation, the dipyridamole particles would distribute homogeneously throughout the entire volume of the well. However, when using orbital shaking for agitation, the dipyridamole particles stayed as lumps at the bottom of the wells and did not move.

Evaluation of Incubators Without Agitation − Are the Different Set-ups Inherently Comparable?

`In order to be sure whether the two agitation methods, orbital shaking and magnetic stirring, give comparable permeation results permeation experiments were conducted without agitation in all set-ups that are described in Section 2.4. In this way, it was investigated if other parameters (e.g. small differences in temperature) would lead to differences and thus render the comparison of the two agitation methods more difficult. P_{app} and Flux values for the permeation experiments without agitation of the 96-well plates are shown in Fig. 2. The corresponding permeation curves (i.e. the cumulative amount of compound permeated over time) can be found in Supplementary material section 5At room temperature (i.e. designated setting 4 in Section 2.4), the permeation was significantly lower as compared to 37 °C for all the studied compounds. At 37 °C (i.e. designated setting 1-3 in Section 2.4), permeation values are mostly non-significantly different, and their means deviate with no more than 1.15-fold for methylene blue and carbamazepine.

Permeation of Model Drugs Under Magnetic Stirring

Fig. 3 shows the P_{app} or flux values for the studied compounds when using magnetic stirring for agitation at 0, 100, and 600 rpm,

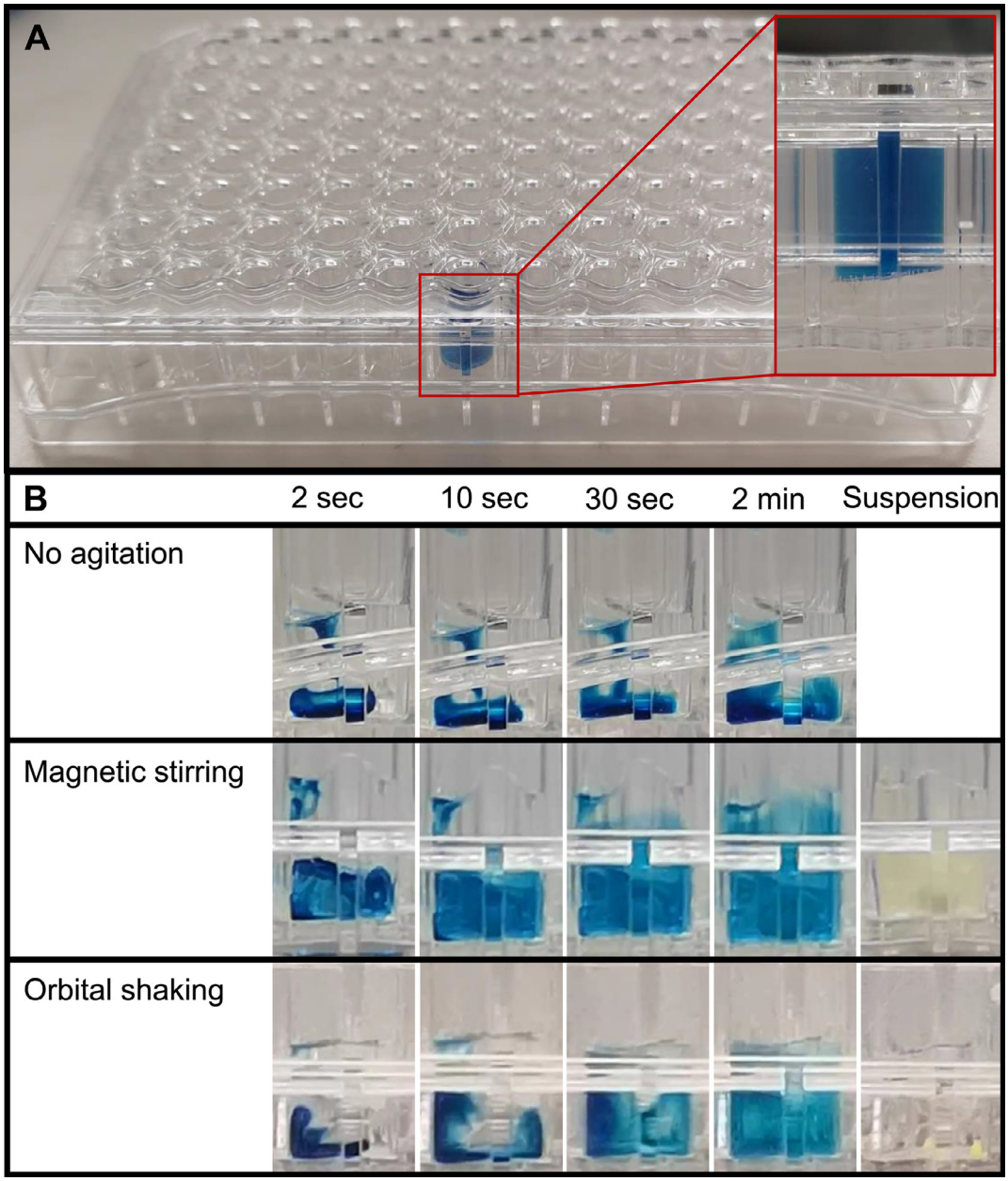

Fig. 1. Visual evaluation of mixing in the 96-well PermeaPlain® plate. A) View of the assembled PermeaPlain® plate with a colored solution filled into a top-well. B) Progress of mixing patterns after 2, 10, 30 or 120 s when using orbital shaking or magnetic stirring or when no agitation was applied. Images of the side of the wells during dilution of a methylene blue solution or a dipyridamole suspension.

respectively. For the readily soluble methylene blue, no statistically significant differences in the permeation were observed between experiments with and without stirring. In contrast, a statistically significant higher permeation was observed for carbamazepine with stirring at 100 and 600 rpm as compared to no stirring. Stirring at 100 or 600 rpm affected carbamazepine permeation equally. In the case of albendazole dispersed in pure buffer, a significantly higher permeation was observed when with increasing stirring rates.

Permeation of Model Drugs Under Orbital Shaking

Fig. 4 shows the P_{app} or flux values of the studied compounds when using orbital shaking at different shaking rates for agitation. In general, orbital shaking did not affect the permeation rates of any of the three studied compounds indicating that the agitation was insufficient to increase the permeation. This is in contrast to the observation in the previous section, where agitation by magnetic stirring did affect the permeation rate of carbamazepine and albendazole.

Permeation of Albendazole in the Presence of TPGS

Albendazole was the drug compound most sensitive to increased agitation by magnetic stirring. Further investigations with 1% (w/w) TPGS in the donor compartment (Fig. 5) revealed that albendazole dispersed in the phosphate buffer containing TPGS showed no significant differences in permeation rate between magnetic stirred or unstirred conditions, in contrast to when TPGS was not added to the buffer. Besides, it is interesting to note that the flux of albendazole in the presence of TPGS increased to the same level as stirring at 600 rpm without TPGS even without magnetic stirring was applied.

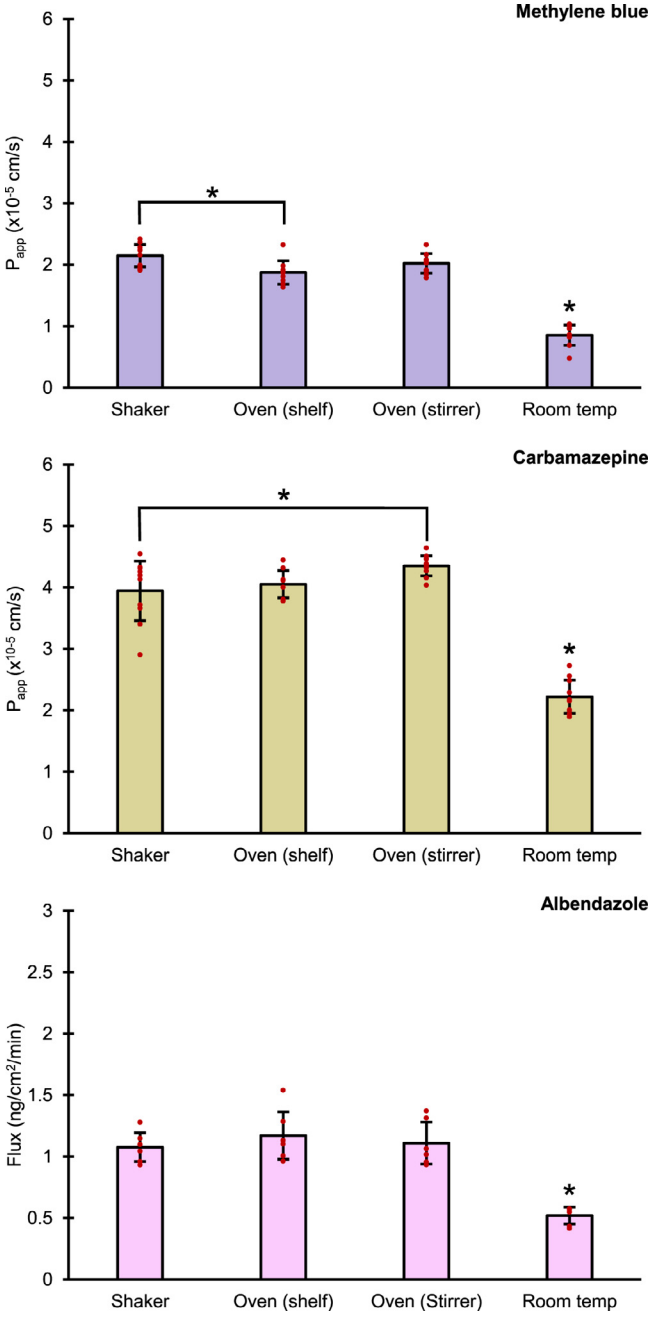

Fig. 2. The apparent permeability (P_{app}) of methylene blue and carbamazepine and flux values of albendazole studied in the 96-well PermeaPlain® plate without agitation. The plates were incubated in a 96-well plate orbital shaker with temperature control at 37 °C (i.e. shaker), a 96-well plate magnetic stirrer placed inside a heating cabinet at 37°C (i.e. oven stirrer), a shelf inside a heating cabinet at 37 °C (i.e. oven, shelf) and on the lab bench (i.e. room temp.). * indicates statistically significant differences. Red dots indicate each replicate (n = 6–10).

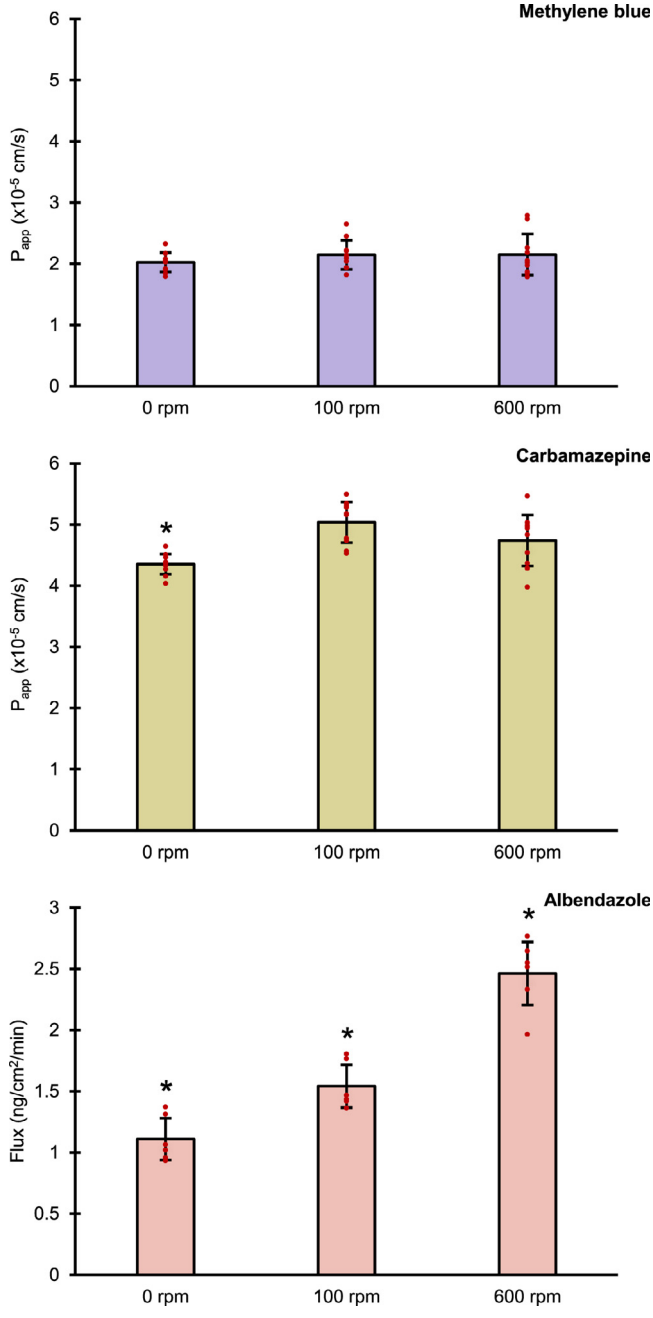

Fig. 3. The apparent permeability (P_{app}) of methylene blue and carbamazepine and flux values for albendazole studied in the 96-well PermeaPlain® plate when using different rates of magnetic stirring for agitation.* indicates statistically significant differences. Red dots indicate the individual replicates (n = 5–10). For easy of comparison, 0 rpm values are presented again in this graph.

Well-to-Well Cross-Talk

When a liquid is added to the bottom compartment of the sandwich plates, it will first fill the space in the bottom, and excess liquid will then get pushed up at the sides of the wells when the middle plate placed on top is displacing the liquid in the bottom compartment (see supplementary material 2). With higher filling volumes, the well-to-well cross-talk risk should increase. Fig. 6 shows the three plates that were filled with a chessboard pattern of water and methylene blue solution after 3 h of incubation in the orbital shaker. All wells in a plate contained the same volume of water and methylene blue solution, but different volumes were tested on each of the three plates (i.e. 250, 300 and 400 μL). Fig. 6 shows that no well-to-well cross-talk was observed in the plate with a filling volume of 250 μL. In contrast, three and ten water wells were contaminated with methylene blue in the plate filled with 300 and 400 μL, respectively. Notably, the contaminated 300 μL wells were only slightly contaminated, as indicated by the very light blue color. In contrast, at a filling volume of 400 μL, several of the contaminated water wells appeared in dark blue shades. All contaminated wells (Fig. 6) are

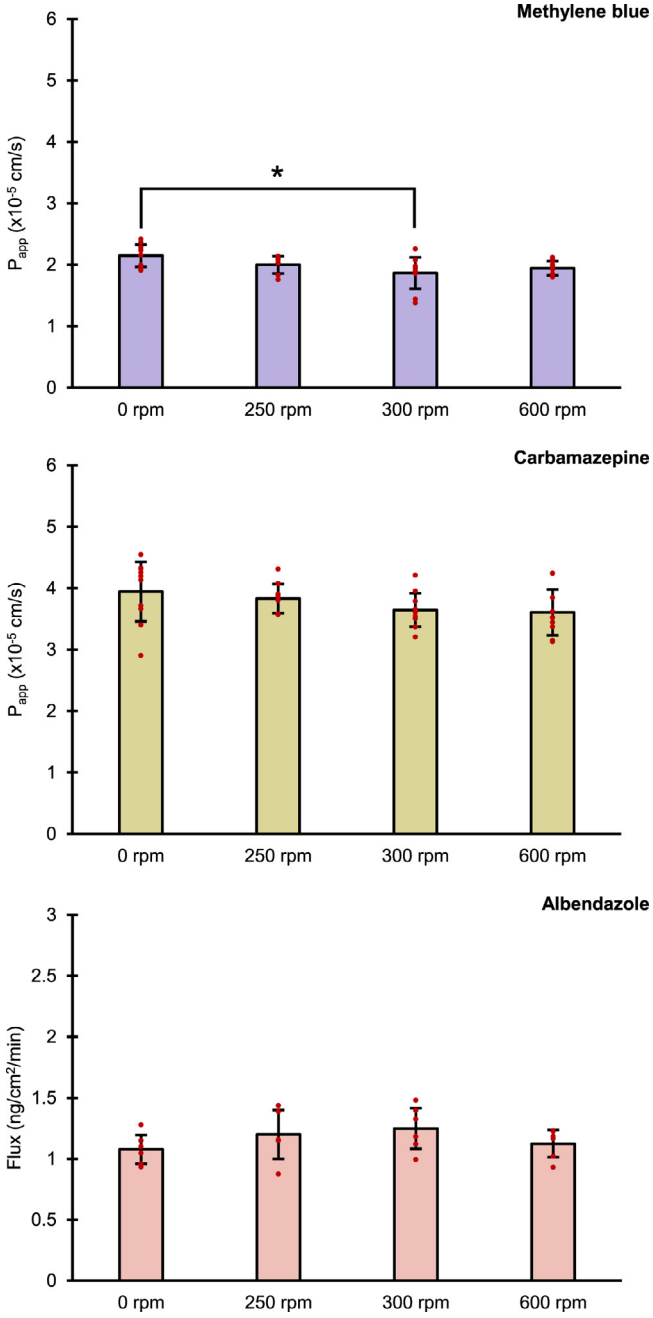

Fig. 4. The apparent permeability (P_{app}) of methylene blue and carbamazepine and flux values for albendazole studied in the 96-well PermeaPlain® plate when using different rates of orbital shaking for agitation. * indicates statistically significant differences. Red dots indicate each replicate (n = 5–10). For easy comparison, 0 rpm values are presented again in this graph.

situated on the edge of the plates indicating that well-to-well cross-talk is more likely for wells at the rim. We have experienced, that the well-to-well cross-talk often is observed even before the plates are agitated.

Too low volumes in the bottom compartment may affect permeation rates by e.g. decreasing the area of the membrane in contact with the donor solution/suspension. Therefore, it was tested if lower volumes of donor medium (i.e. down 250 μL) would affect the permeation rates. The permeation rates were not reduced by reducing the donor volume to 250 μL (see supplementary material 3).

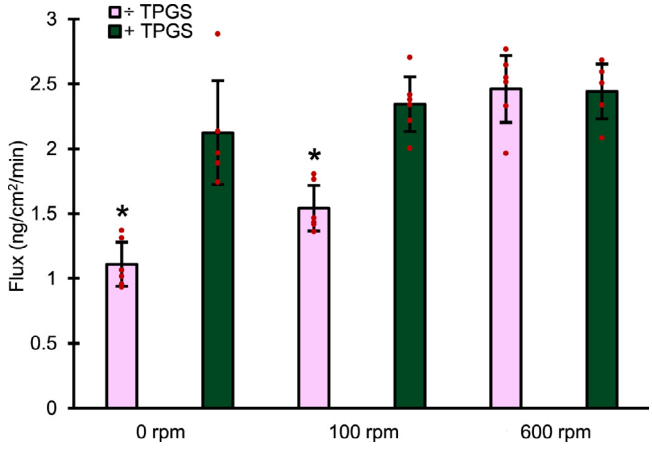

Fig. 5. The apparent flux values for albendazole studied in the 96-well PermeaPlain® plate when using different rates of magnetic stirring for agitation with the presence of 1% TPGS in the donor compartment. * indicates statistically significant differences. Red dots indicate each replicate (n = 5–6). For easy comparison, 0 rpm values and flux values in absence of TPGS are presented again in this graph.

Discussion

Orbital Shaking Resulted in Poor Mixing

Evaluating orbital shaking as an agitation method, the mixing of liquid in the wells of the 96-well sandwich plates was minimal when exposed to orbital shaking. Despite the fact that geometry of the 96-well sandwich plates used here is substantially different from other containers, it is interesting to note, that Buch et al. observed similar tendencies when mixing Erlenmeyer flasks using orbital shaking.[24] They found that the bulk of the liquid either could be "in-phase" with the shaking table resulting in adequate agitation of the liquid within the flask or "out-of-phase", where only a minimal fraction of the bulk was rotating along with the shaking, and the liquid within the flask was poorly mixed. They found that the transition between in-phase and out-of-phase mixing could occur from small changes in experimental conditions such as shaking diameter, shaking volume, shaking speed, and viscosity of the liquid. They developed a model to predict if a liquid during shaking would be in or out of phase based on acceleration patterns and interferences. Despite the obvious differences in the geometry, the hypothesis has been successfully applied to other types of 96-well plates.[24,25] One should bear in mind, that the aforementioned hypothesis was developed for orbital shaking of containers with a round shape, and the model is highly dependent on the diameter of the container. In contrast, the bottom wells of the PermeaPlain® 96-well plate have a substantially different geometry: they are not round, and hence a diameter cannot be given. Therefore, the model cannot directly be applied to the plates used in this study. Still, when looking at the permeation results (Fig. 3) and the visual evaluation of mixing (Fig. 1), it appears appropriate to conclude that liquids in the bottom plates never were in-phase during orbital shaking. Approaches to overcome this challenge may include increasing shaking rate, or the diameter of shaken orbitals. However, increasing the volume or increasing the shaking rate appears not appropriate due to the risk of well-to-well cross-talk as discussed in detail below.

Suggested Agitation Conditions for Optimum Permeation

Dahlgren et al. compared in vivo permeability values (P_{eff}) with in vitro P_{app} values from Caco-2 cell studies of 13 compounds and found

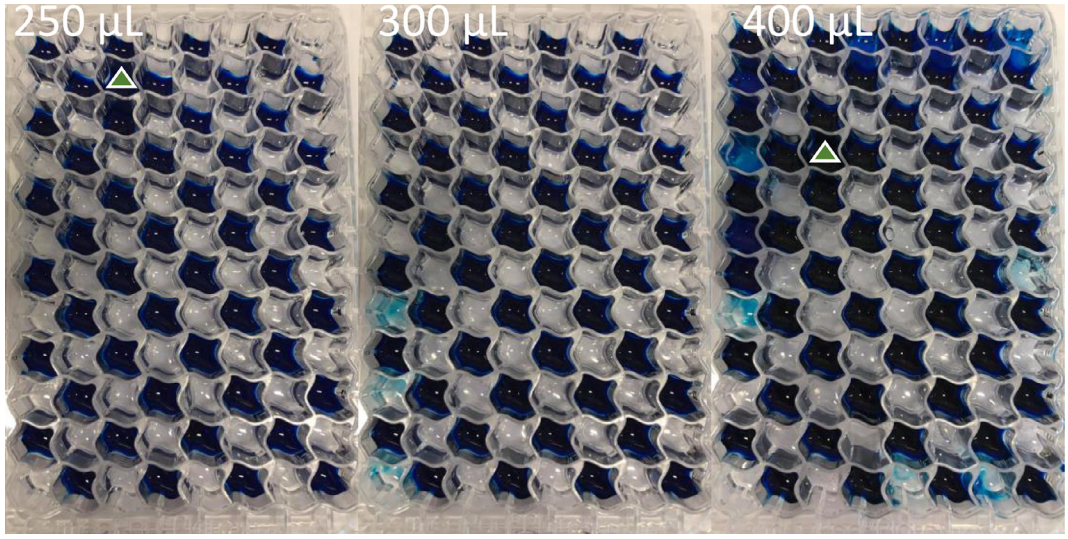

Fig. 6. Images of PermeaPlain® plates filled with 250, 300, or 400 μL methylene blue solution and water in all wells in a chessboard pattern after 3 h of incubation in a 96-well plate orbital shaker. Green triangles indicate wells that were mistakenly filled with methylene blue instead of water.

that the P_{app} in general was about 10-fold lower than P_{eff} for the studied compounds[26]. It has been shown that efficient removal of the dissolved drug via permeation has an impact, i.e. it slowed down the precipitation from supersaturated systems by lowering the degree of supersaturation in the dissolution compartment[7,8], and recent studies have indicated that the extent of permeation leads to an even more potent precipitation inhibition[27]. These assumptions lead to the desire for agitation conditions that support high permeation rates. Furthermore, higher concentrations in the acceptor compartment are easier to quantify.

In this study, orbital shaking, at any rate, did not affect the permeation of any of the three model compounds. In contrast, magnetic stirring enhanced the permeation, at least that of the poorly soluble and lipophilic drug compounds, carbamazepine (minor influence) and albendazole (major influence). It was assumed that poor mixing conditions affect poorly water soluble and lipophilic compounds more or in other words these compounds profit more from proper mixing than water soluble compounds, which corresponds to findings in the literature for other models[12,17]. Therefore, we assumed that the plates were not agitated well enough by orbital shaking to enhance the permeation. Avdeef et al. observed similar difficulties with shaking of parallel artificial membrane permeability assay (PAMPA) plates[18]. Similar to the current study, in Avdeef and co-workers' study, magnetic stirring was found more effective (of note: in Avdeef's study and the current study, stir bars rotated about a vertical axis and a horizontal axis, respectively). They later suggested that the resistance of the unstirred water layer is more than 3-fold lower in the wells on the edge of a PAMPA plate than for the central wells[28]. In this study, we observed that the variations within each study were randomly distributed all over the plates.

If exclusively regarding this study's permeation results, magnetic stirring seems superior over orbital shaking due to the easily obtained permeation enhancement. However, challenges may arise during magnetic stirring: In our hands, two magnets placed in adjacent wells are likely to attract each other and stop stirring, making only half of the wells in a plate available for experiments (see supplementary material 1). Another challenge is that the magnetic stirrer used in this study is producing heat by the motor. The wells exposed to stirring at 600 rpm showed a temperature increase of approximately 3 °C during the 6 hours of the experiment (see supplementary material 4). This may represent a severe drawback of the magnetic stirring since the permeation rate is highly temperature dependent.

Surfactants in the Donor Compartment are Counterbalancing the Effects of Insufficient Agitation.

The presence of solubilizing components (e.g., micelles and cyclodextrins) in the donor compartment during permeation studies decreases the apparent permeation of drug compounds. Consequently, overdosing solubilizing components will result in decreased absorption.[6,29] However, the process is more complex when the drug is kept in suspension, as the amount of molecularly dissolved drug is not lowered by the solubilizers. In the case of fasted state simulated intestinal fluid (FaSSIF), Frank and colleagues did not observe any significant changes in the flux of ABT-102 even though FaSSIF increased the apparent solubility by 30-fold.[30] In comparison, Ingels and colleagues showed that danazol suspensions were able to show a significant increase when FaSSIF was present in the donor.[31]

The permeation of poorly water soluble drug compounds in the presence of cyclodextrin has in independent studies been demonstrated much more similar at different stirring rates when cyclodextrin was present compared to when it was not. This has led to the general conclusion that cyclodextrin was able to decrease the apparent thickness of the UWL.[32,33] In contrast, recent studies found that the passive diffusion of drug compounds was hampered within the UWL when solubilized by cyclodextrin or surfactants.[34]

In this study, we observed an enhancement of the flux in the presence of TPGS, and, similar to the cyclodextrin study, the effects of stirring seemed to be counterbalanced by the surfactant. We consider further proof necessary to correlate the observed effects directly to a decrease in the unstirred water layer, which is beyond the scope of this study.

Well-to-Well Cross-Talk

The term well-to-well cross-talk describes the process of content fractions of one well moving to another adjacent well during experiments. The systematic investigation in this study demonstrated that well-to-well cross-talk is more likely to occur in wells at the rim of a plate than in wells placed in the center. At this time, we cannot explain why the cross-talk occurred preferably at the rim. However, during the study, we observed that methylene blue solutions in

many cases had moved from one well to another during assembling or moving the plates around, which is even before starting to shake the plates. An explanation for the well-to-well cross-talk at the rim of plates could be that the top wells at the edge can be slightly distorted, thereby displacing a higher volume of the medium in the bottom wells. High-velocity shaking speeds can still cause or increase the cross-talk, which was observed at 600 rpm with significant cross-talk between wells filled with 250 μL of medium (data not shown). To reduce the risk of well-to-well cross-talk, the current study demonstrated that preferably volumes below 400 μL in the donor compartment of PermeaPlain® plates (and Permeapad® plates having the same design) should be applied.

Conclusion

In this study, we systematically compared orbital shaking and magnetic stirring for agitation of a novel 96-well sandwich plate. The study revealed that orbital shaking (under the conditions tested) did not mix the liquids in the donor wells efficiently, and in turn the permeation of compounds from the bottom compartment to the top compartment was virtually unaffected by orbital shaking as compared to non-agitated conditions. On the other hand, magnetic stirring was found suited to mix the liquids in the wells efficiently, and in turn the permeation of the compounds was found enhanced as compared to unstirred conditions. The observed stirring effect was most pronounced for the most lipophilic among the three compounds investigated, Although direct comparison is difficult due to the different geometry of the sandwich-inserts, these observations are in congruence with earlier studies on other types of sandwich plates, e.g. PAMPA Transwell® plates, as well as the UWL-hypothesis. Interestingly, the impact of stirring upon permeation of the most lipophilic compound was less pronounced again in the presence of a solubilizing surfactant. It could be demonstrated that undesired well-to-well cross-talk may be minimized/avoided by carefully selecting the volume of the donor solutions in the bottom plate.

Declaration of Competing Interest

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

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Supplementary Materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.xphs.2021.06.006.

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