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Research paper

Permeability assessment for solid oral drug formulations based on Caco-2 monolayer in combination with a flow through dissolution cell

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Abstract

The aim of our study was to develop an apparatus assessing *in vitro* permeation through Caco-2 monolayers of oral solid dosage forms as a possible tool to forecast *in vivo* performance. Therefore, flow through dissolution and permeation modules were connected by means of a stream splitter. Permeation was measured in a specially designed cell, dissolution took place in the apparatus 4, USP. In order to test the apparatus for its reproducibility and conclusiveness, different tablet strengths and varying release profiles of propranolol HCl tablets were produced and evaluated. It was shown that for both tablet species, immediate and extended release, the apparatus was able to measure permeation through Caco-2 monolayer as well as dissolution simultaneously with high precision and reproducibility. The permeated amount of the three immediate release tablets with increasing dosage strength showed linear dependency on the dosage strength. Furthermore, the effect of retarded release on permeation could be detected and conclusive data for dissolution and permeation were obtained. In summary, connecting cell culture based permeability assessment with compendial flow through dissolution equipment led to promising results and poses the base for more advanced studies for detecting influences of dosage forms on permeation process.

Keywords: Oral absorption; Caco-2 cells; Dissolution; Permeation; Propranolol HCl; Biopharmaceutical classification system (BCS)

1. Introduction

For the assessment of permeability of drugs across the intestinal mucosa, the so-called Caco-2 cell model is extensively used and generally accepted as a surrogate to predict oral absorption [1–3]. However, such permeability assays are normally carried out with pure and completely dissolved compounds, and thus lack the possibility to monitor influences of the dosage form and excipients on permeability. Excipients opening tight junctions of intestinal epithelia, and thus enhancing permeation of passively, transcellularly absorbed drugs, are described in the literature [4,5]. Furthermore, effects of excipients (Tween 80, vitamin E TPGS, etc.) on permeation via interaction with intestinal cellular efflux systems are reported in the literature [6–11]; whereas it is crucial in these cases to assess *in vitro* Caco-2 permeability with *in vivo* relevant concentrations as recently stated by Brouwers et al. [11]. These interactions of excipients interfering with permeation processes can lead to unexpected results in formulation development if *in vitro* dissolution tested oral solid dosage forms are administered in animal or human studies.

The assessment of intestinal permeability for drugs formulated as solid oral dosage forms may be possible if one succeeds to connect in an appropriate way a setup for permeability measurement (e.g. Caco-2 cell monolayers) with a setup for dissolution testing (e.g. paddle method, flow through cell, etc.). Several approaches for such

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combinations have already been described. A continuous dissolution/Caco-2 system to predict the dissolution–absorption relationship using a paddle apparatus has been described by the group of Polli [12]. Other groups have been reported to work on open systems for simultaneous determination of dissolution and permeation [12–17]. However, these systems exhibit some drawbacks, e.g. they are primarily designed to measure dissolution and permeation of plain powders. Additionally, employment of non-compendial dissolution equipment may limit comparability of dissolution data and low flow rates in the dissolution module may not be physiologically relevant.

To overcome these limitations, we considered the flow through apparatus 4 as described in USP ⟨724⟩ as a useful starting point, as it allows the employment of complete dosage forms under highly reproducible conditions and the application of physiologically relevant flow rates. However, to perform permeability measurements with Caco-2 monolayers under flow through conditions rather than in the conventional static way (i.e. Transwell® in well plate), an appropriate flow through permeation cell (FTPC) had to be developed. Furthermore, to connect the dissolution with the permeation cell, a stream splitter had to be implemented to allow the necessary adjustment of different flow rates in the two modules in the new apparatus.

Prior to testing complete dosage forms, it was investigated if the flow through permeation cell (FTPC) provides comparable results for permeation data compared to static experiments performed in conventional Transwell® systems. For this purpose, two different compounds were tested: propranolol as a high permeability marker (marker for unstirred water layers) and fluorescein-Na as a low permeability marker (marker for barrier integrity). For the permeation module, several criteria have to be matched such as: (i) the integrity of the Caco-2 monolayer has to be maintained throughout the experiments and (ii) conditions for permeation should be comparable to those in Transwell® systems. Since the dissolution media are getting directly in contact with the Caco-2 monolayer, KRB was used for both dissolution and permeation media.

Propranolol HCl was chosen because of its biopharmaceutical properties: It shows high permeability in Caco-2 transport experiments [18] and human perfusion studies [19]. Caco-2 experiments indicate that propranolol HCl permeates transcellullarly, and neither has affinity to cellular efflux or to influx systems [18,20,21]. In addition, propranolol HCl is highly soluble in aqueous buffers [18,22] and hence is assigned to BCS class I [18,23,24].

In order to test and validate the newly designed combined apparatus, propranolol HCl released from a solid dosage forms at different dosage strengths was used. Beyond that, extended release propranolol HCl tablets with varying amounts of Eudragit® NE 30D as a retarding agent were produced. For propranolol, increase of dosage strength should lead to a direct proportional increase of permeation; retardation of release should lead to a direct change of the appearance rate in the *trans*-mucosal receptor compartment. Up to now, no other flow through *in vitro* setup has been described that would allow to monitor the impact of such formulation related parameters on the intestinal permeability of a drug formulated as solid oral dosage form.

2. Materials and methods

2.1. Materials

Transwell® permeable filter inserts (pore size 0.4 μm, 1.13 cm^2, Transwell® type 3460) were purchased from Corning Incorp. Life Sciences (Acton, MA). Dulbecco's modified Eagle's medium (DMEM), non-essential amino acids (NeAA) and fetal bovine serum (FBS) were purchased from GIBCO (Invitrogen Corp. Carlsbad, CA). Fluorescein-Na was purchased from Sigma–Aldrich (St. Louis, MO) and propranolol HCl from Synopharm GmbH & Co KG (Barsbüttel, Germany). Organic solvents were of HPLC grade. Eudragit® NE 30D was a kind gift from Degussa (Röhm Pharma Polymers, Darmstadt, Germany). Avicel PH 102 was kindly donated by Lehmann and Voss (Hamburg, Germany) and Lactose EP type D20 by J.A. Meggle Milchindustrie (Reitmehring, Germany); PVP insol. was a kind gift from BASF (Ludwigshafen, Germany) and Aerosil 200 was kindly donated by Degussa (Rheinfelden, Germany); Mg-stearate was from Synopharm GmbH & Co KG (Barsbüttel, Germany). All tablet excipients were of Ph. Eur. grade. Composition of Krebs–Ringer Buffer (KRB) was as follows: 1.41 mM $CaCl_2$, 3.00 mM KCl, 2.56 mM $MgCl_2$, 142.03 mM NaCl, 0.44 mM K_2HPO_4, 4.00 mM D-glucose and 10.0 mM Hepes. KRB was adjusted to pH 7.4 by means of NaOH. All salts for KRB were of cell culture tested grade and obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Cell culture

Caco-2 cells, clone C2BBe1, were purchased at passage 60 from American Tissue Culture Collection (ATCC; Manassas, VA) and used at passages 70–92. Caco-2 cells were grown to ~90% confluency in 75 cm^2T-flasks with DMEM supplemented with 10% FBS and 1% non-essential amino acids (NeAA). Culture medium was changed every second day. The incubator temperature was set to ~37 °C in an atmosphere of ~85% relative humidity and ~5% CO_2. After trypsinisation, cells were seeded on Transwells® at a density of 60,000 cells/cm^2. Transepithelial electrical resistance (TEER) was measured routinely (EVOM, World Precision Instruments, Berlin, Germany) and only monolayers with a TEER > 350 Ω cm^2, with background for plain filters subtracted, were used for transport studies.

2.3. Transport assay

Caco-2 monolayers were used 21–25 days post seeding. Prior to transport experiments, DMEM with supplements

was removed and KRB, pH 7.4, was added to the apical and basolateral compartment. Apparent permeability (P_{app}) was calculated according to the following equation

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{A \cdot c_0} \quad (1)$$

where A (cm^2) is the nominal surface area of the monolayer and c_0 (μg/ml) is the donor concentration at $t = 0$ h. The ratio dQ/dt was calculated from the slope of the linear part of the mass increase in the acceptor compartment. Concentration of propranolol HCl in the donor compartment was 30 and 10 μg/ml for fluorescein-Na, respectively. Integrity of the Caco-2 monolayers was checked microscopically and by measuring TEER values before and after the experiments. Donor solutions were pumped into the FTPC with a flow rate of 1.0 ml/min and samples were withdrawn from the circulating basolateral compartment after 30, 60, 90, 120, 150, 180 and 210 min. Retrieved volumes were replenished with fresh KRB ($n = 5$ for both compounds). The retrieved amount of propranolol was taken mathematically into consideration.

2.4. Preparation of tablets

Immediate release tablets (IR tablets) were manufactured using direct compression (for formulation see Table 1). Ingredients were sieved (mesh size 315 μm), blended in an turbula mixer (TS Bachofen, Basel, Switzerland) and subsequently compressed using a Korsch EK 01 (Berlin, Germany) eccentric tablet press gaining tablet mass of 100 mg and crushing strength of 100 N (Erweka hardness tester, type TBH 30 M, Erweka, Heusenstamm, Germany).

Extended release tablets (ER tablets) were prepared as follows (for formulation see Table 2): lactose, Avicel and propranolol HCl were granulated using a 30% dispersion of Eudragit® NE 30D. Water was added until a capable granulate was formed. After sieving through a 2 mm sieve, the wet powder was dried for 1 h in an oven with circulating air at 45 °C followed by an over night drying at room temperature. Shortly before compressing, freshly sieved Aerosil 200 and Mg-stearate were added and blended for 5 min in a turbula mixer. Tablets were produced with a mass of 100 mg and a crushing strength of 100 N. For the determination of the content uniformity, tablets were extracted with 1 M HCl and the filtered supernatant was analyzed by HPLC after appropriate dilution ($n = 10$).

Table 1
Composition of the immediate release propranolol HCl tablets

	5 mg (%)	10 mg (%)	20 mg (%)
Avicel pH 102	70	70	70
Lactose EP type D20	23	17	7
PVP insol.	1	1	1
Aerosil 200	1	1	1
Mg-stearate	1	1	1
Propranolol HCl	5	10	20

Table 2
Composition of the 10 mg propranolol HCl extended release tablets

Eudragit® NE 30D[a]	4%	8%
Avicel PH 102	55%	55%
Lactose EP type D20	29%	25%
Propranolol HCl	10%	10%
Water	q. s.	q. s.
Aerosil 200	1%	1%
Mg-stearate	1%	1%

[a] Calculated as solid amount of a 30% dispersion.

2.5. Analytics

Quantification of propranolol HCl was performed using a Dionex HPLC system, implemented with a RP 18 (LiChroSpher® 100, Merck), 5 μm, 12.5 cm column. The HPLC system consisted of a Dionex P580 pump, a Dionex ASI 100 auto sampler, a UVD 170 S detector and a Dionex STH 585 column oven. Software was Chromeleon® 6.60, SP 1 build 9.68. Mobile phase was 45% (v/v) water, 22% (v/v) acetonitrile, 33% (v/v) methanol, 0.033% (v/v) triethylamine and 0.044% (v/v) phosphoric acid. Flowrate was 1.2 ml/min and oven temperature was 40 ± 1 °C. Detection was done with UV at 215 nm, whereas linearity ($R > 0.999$) was given between 30 ng/ml and 100 μg/ml. Retention time was 3.00 ± 0.15 min. It was shown that none of the excipients interfered with the analysis.

Quantification of fluorescein-Na was performed using a Cytofluor II fluorescence reader ($\lambda_{exc} = 485$ nm, $\lambda_{em} = 530$ nm) with Cytofluor software version 4.2 (PerSeptive Biosystems, Wiesbaden-Norderstedt, Germany). Measurement took place in a 96-well plate and was done after addition of 150 μl of 10 mM NaOH to 50 μl analyte. Linearity ($R > 0.999$) was given between 5 ng/ml and 1 μg/ml.

2.6. General consideration in respect to the apparatus

The apparatus can be divided into two modules: a flow through permeation module and a flow through dissolution module, whereas both modules are connected by means of a stream splitter (Fig. 1). In the permeation module, flow rate was set to 1.0 ml/min and in the dissolution module to 6.5 ml/min. The flow through permeation module can be subdivided into two compartments: the open apical and the closed basolateral compartment with a Caco-2 monolayer upon a Transwell® mounted between these both compartments. Within the basolateral compartment, a magnetically stirred glass vessel was installed for sampling and for setting up a supply for KRB buffer. Sampling was performed at three different locations, designated in Fig. 1 as "D" – dissolution, "A" – apical and "B" - basolateral. The flow through dissolution module consisted of the USP apparatus 4 (flow through cell, Sotax CE1, Sotax, Germany) equipped with a 12 mm dissolution cell and a filter preventing undissolved solids from escaping. Tablets were placed upon small glass beads as described in ⟨724⟩ of USP [25]. Samples taken at "D" represent the

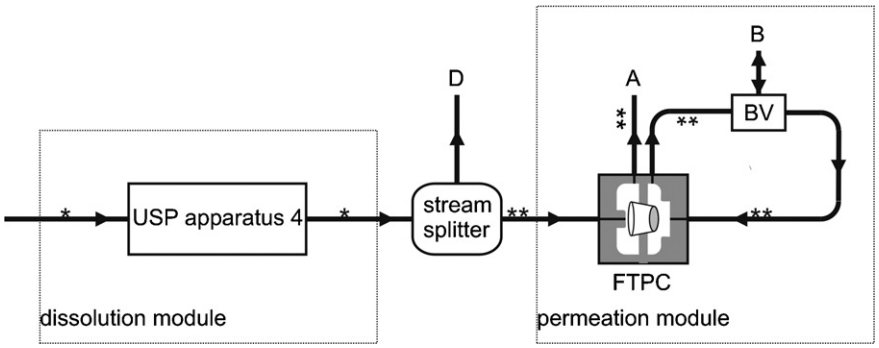

Fig. 1. Schematical depiction of the apparatus for combined flow through measurement of dissolution and permeation (*flow = 6.5 ml/min; **flow = 1.0 ml/min). Sampling ports are indicated with capitals: D, sampling port dissolution; A, sampling port apical, B, sampling port basolateral.

dissolution process, samples collected at "A" represent the amount and extent of drug, which appeared in the apical compartment of the FTPC being available for permeation through the Caco-2 monolayer (Sample volume at "D" was 0.5 ml, and 150 μl at "A"). Since the basolateral compartment of the permeation module is a closed compartment, the retrieved samples were replenished with blank KRB buffer (sample volume at "B" was 150 μl). The complete volume in the basolateral compartment was calculated and experimentally confirmed as 10.5 ml. Dissolution as well as permeation took place in KRB, pH 7.4 ± 0.1. Prior to each experiment, KRB was degassed using filtration through a 0.22 μm filter and by means of ultrasonic. In order to avoid any issues concerning adsorption and biocompatibility, the tubing (ID 1.0 or 2.0 mm) consisted of Teflon®, and the FTPC and the stream splitter were made of PEEK (polyetheretherketone). All streams in the apparatus were driven by membrane dosage pumps of type Stepdos 03® (KNF Neuberger, Freiburg, Germany) in combination with pulsation dampers. The apparatus 4 and the flow through permeation cell were submersed in a water bath and heated to 37.0 ± 0.5 °C. Prior to each experiment, Caco-2 monolayers were washed twice with KRB and equilibrated with KRB for at least 30 min in an incubator. After that, transepithelial electrical resistance (TEER) was measured, the Transwell® was subsequently inserted in the FTPC, and cells were allowed to equilibrate for another 15 min with an apical and basolateral flow of each 1.0 ml/min. After the experiments, Transwells® were taken out of the FTPC and integrity of the Caco-2 monolayer was confirmed microscopically and again by TEER measurement.

2.7. Data treatment

All data are given as means ± standard deviation. Statistical analysis has been done using Sigma Stat for Windows version 3.0.1 build 3.01.1.

In order to compare the different concentration time profiles, mean times of the dissolution and permeation curves have been calculated. Accordingly, mean times for the different sampling ports were calculated

$$\text{mean time at D}: \mathrm{MT_D} = \frac{(t \cdot m_\mathrm{D}) - \mathrm{AUC_D}}{m_\mathrm{D}} \quad (2)$$

$$\text{mean time at A}: \mathrm{MT_A} = \frac{(t \cdot m_\mathrm{A}) - \mathrm{AUC_A}}{m_\mathrm{A}} \quad (3)$$

$$\text{mean time at B}: \mathrm{MT_B} = \frac{(t \cdot m_\mathrm{B}) - \mathrm{AUC_B}}{m_\mathrm{B}} \quad (4)$$

t is the time duration over which dissolution or permeation was measured, m_D and m_A are the masses which cumulatively appeared at the respective sampling port after time t, m_B is the mass which permeated and was detected at sampling port B after time t. $\mathrm{AUC_D}$, $\mathrm{AUC_A}$ and $\mathrm{AUC_B}$ are the areas under the amount time curves for the respective sampling ports (all areas were calculated using trapezoidal rule).

For regression, Sigma Plot 2004 for Windows Version 9.0 was used (regression values are given as means ± SD error).

3. Results

3.1. Effects of flow rate in the FTPC on P_{app} of propranolol and fluorescein-Na

P_{app} values of propranolol HCl and fluorescein-Na were assessed with the FTPC. For an apical flow rate of 0.75 ml/min, P_{app} of propranolol HCl was determined as 2.2×10^{-5} cm/s. Apical flow rate of 1.25 ml/min led to a P_{app} of 2.9×10^{-5} cm/s, flow rate of 1.00 ml/min led to a P_{app} of $2.5 \pm 0.52 \times 10^{-5}$ cm/s ($n = 4$). At 1.00 ml/min, P_{app} of fluorescein-Na was 4.7×10^{-7} cm/s. For the flow rates of 0.75–1.25 ml/min, TEER values measured after the experiments remained above 350 Ω cm^2 and monolayer were microscopically intact. Flow rates above 2 ml/min showed deleterious effects on Caco-2 cell monolayers.

3.2. Assessment of drug permeability and dissolution at different dosage strengths

Three immediate release tablet batches with increasing amount of propranolol HCl were investigated. The concentration time profile measured at sampling port D and at A

is shown in Fig. 2A and B. The dissolved amount after 120 min calculated by means of trapezoidal rule yielded for all three batches 90% of the labelled amount or more (not shown). Five and ten milligrams IR propranolol HCl tablets showed a rather rapid release of propranolol and exhibited a peak concentration at approximately 10 min at sampling port D and at 15 min at sampling port A. The 20 mg immediate release propranolol HCl tablets showed a slightly delayed initial release of propranolol HCl with a shoulder and subsequently reached maximum peak concentration at about 23 min at sampling port D and approximately 30 min at sampling port A.

In Fig. 2B, a sigmoid time curve of permeated propranolol HCl was observed. Since the volume of the basolateral compartment was experimentally determined as 10.5 ml, the permeated amount could easily be assessed from the measured concentrations. The permeated amount after 120 min was significantly different for all propranolol HCl tablets (ANOVA, Holm–Sidak test, $p < 0.001$).

3.3. Assessment of drug permeability and dissolution at different release kinetics

Three different tablet species with differently retarded 10 mg propranolol HCl were investigated. Retardation was mediated by increasing amounts of Eudragit® NE 30D. Note that sampling time was prolonged for the extended release tablets from 120 to 180 min. The concentrations at sampling port D and A are shown in Figs. 3A and B. Concentration profiles of the immediate release and the two retarded tablet species can easily be distinguished from each other since retardation led to decreased

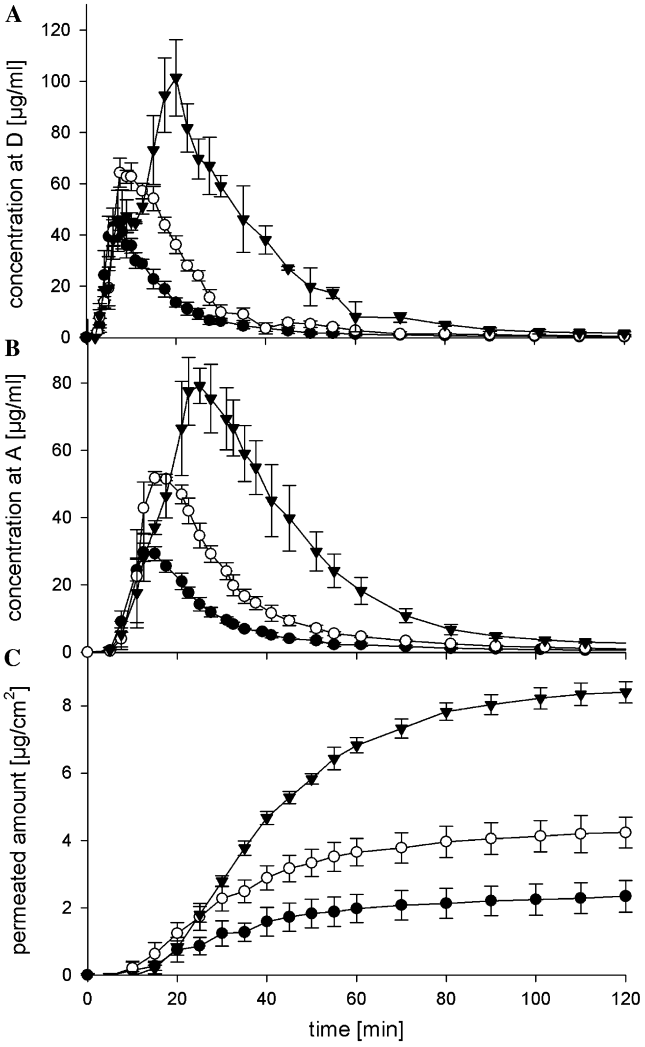

Fig. 2. Concentration time trends for immediate release tablets at sampling port D and A (A and B). (C) Shows the permeated amount calculated from the concentrations assessed at sampling port B. Closed circle (●) represent 5 mg, open circles (○) represent 10 mg and triangles down (▼) represent 20 mg immediate release propranolol HCl tablets (means ± SD, $n = 4$–5 for each dosage strength).

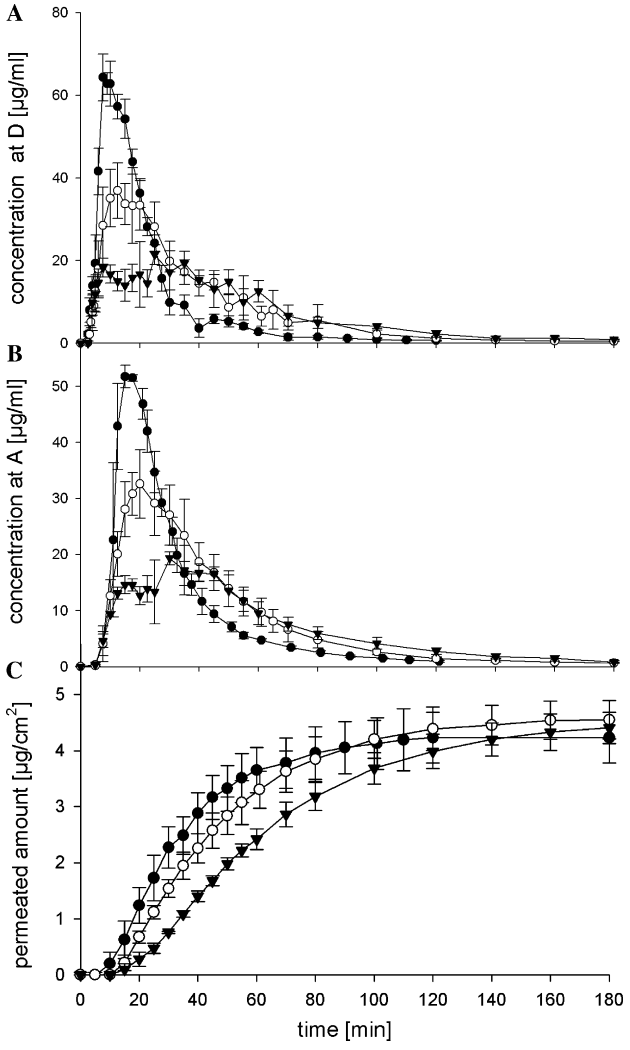

Fig. 3. Concentration time trends for extended release tablets at sampling port D and A (A and B). (C) Shows the permeated amount calculated from the concentrations assessed at sampling port B. Closed circles (●) represent the 10 mg propranolol HCl immediate release tablet, open circle (○) represent the 10 mg extended release tablet with 4% Eudragit® NE 30D and triangles down (▼) represent the 10 mg extended release tablet with 8% Eudragit® NE 30D (mean ± SD, $n = 4$–5).

maximum concentration and flattened peaks. The cumulative release after 180 min based on the AUC was for each, the immediate release and the 4% Eudragit® NE 30D containing extended release tablet, greater than 90 %, whereas the 8% Eudragit® NE 30D containing extended release tablet released about 82% of the labelled amount within the time of the experiment. In summary, the addition of Eudragit® NE 30D led to an evident, concentration dependent retardation of release of propranolol HCl. Obviously, and as expected for a highly permeable compound, the appearance of propranolol HCl in the basolateral compartment of the permeation module is governed by its concentration at the apical side of the permeation device which depends on the release rate from the dosage form (Fig. 3C).

4. Discussion

4.1. Rationale for the particular setup and choice of media, flow rates and formulations

To allow the option of different media and flow rates, the dissolution and permeation module requires the use of a stream splitter which supplies the permeation module with appropriate low flow rates to ensure viability and integrity of Caco-2 monolayers. Since the stream splitter works depressurized, effects on the dissolution in the flow through dissolution cells are highly unlikely and have not been detected. Both modules are hydrodynamically separated, and thus can be run independently. Therefore, pressure variations in the dissolution module possibly caused by pumps and clogged filters do not carry over into the permeation module where pressure sensitive Caco-2 cell monolayers are located. The rationale to implement a circulating basolateral compartment are the higher concentrations compared to those in an open compartment, i.e. facilitating analytics of the permeated drug.

Working with sensitive cell culture models such as Caco-2 cell monolayers puts limitations on the use of the aqueous media to be in contact with them. Viability and integrity of a Caco-2 monolayer depends, just to mention some important parameters, on isosmotic pressure in combination with appropriate pH values, and thus choice of dissolution media is very limited when the liquid with the dissolved drug is directly led over the Caco-2 monolayer. Although it has been reported that HBSS-like buffers tend to underestimate solubility of drugs due to its high hydrophilicity and its excessive content of possible counter-ions [26], KRB was chosen as both dissolution and permeation buffer since for the model drug propranolol HCl no solubility issues are expected. The pH for dissolution and permeation measurement was chosen to be 7.4, although pH in the small intestine is reported to be one magnitude lower. However, the authors wanted to avoid any issues arising from false efflux phenomena for weak bases as it has been described by Neuhoff et al. [27]. Moreover, applying cell culture compatible biorelevant dissolution media was beyond the scope of this first evaluation of our new apparatus.

The flow rate in the dissolution module was set to 6.5 ml/min for two reasons: first, volumes in the small intestine accessible for dissolution of orally administered drugs are generally regarded to be between 120 and 350 ml for the fasted state and up to 1600 ml for the fed state [28–30]. For the here described setup, an experiment time of 120 or 180 min combined with a flow rate in the dissolution module of 6.5 ml/min yielded total volumes of 780 or 1170 ml and are ranging within the above-mentioned physiological limits. The second reason was that concentration of propranolol HCl resulted in a physiologically relevant magnitude. Assuming an administration of 10 mg of propranolol HCl dissolving in 200–250 ml of intestinal fluid, a concentration of 40–50 μg/ml would be yielded. Maximum concentrations in the apparatus for the 10 mg immediate release tablet of 50–60 μg/ml do fit well to the estimated values.

For selection of the flow rates in the permeation module, the determination of P_{app} was essential. For comparison, the P_{app} obtained with a flow rate of 1 ml/min in the FTPC and the P_{app} obtained with static conditions as reported in the literature are shown in Table 3. Since permeation of highly permeable compounds, such as propranolol HCl, may be limited by stagnant water layers adhering to membrane surfaces [31], a comparable P_{app} for propranolol HCl is of vital interest in order to assure comparable hydrodynamic parameters. P_{app} for propranolol found in the FTPC ($2.5 \pm 0.52 \times 10^{-5}$ cm/s) nicely falls within the range reported in the literature and thus suggests that both assays deliver comparable results for highly permeable compounds and both systems exhibit comparable hydrodynamical properties. For fluorescein-Na, a low permeability compound, a comparable P_{app} ($4.7 \pm 0.57 \times 10^{-7}$ cm/s) suggests an intact monolayer throughout 210 min. This statement is furthermore compounded by TEER values still being above 350 Ω cm^2 and by microscopical observation of the monolayer. Consequently, apical and basolateral flowrate of 1.00 ml/min was found to be optimal for our purposes since this flow was still high enough to carry over the faster dissolution signal into the apical permeation site and maintained the barrier integrity of the Caco-2 monolayer.

Table 3
Comparison of P_{app} values

	FTPC (cm/s) ($n = 4$)	P_{app} reported in literature (cm/s)
Propranolol HCl	$2.5 \pm 0.52 \times 10^{-5}$	$1.1 \pm 0.5 - 4.3 \pm 0.4 \times 10^{-5}$ [18]
Fluorescein-Na	$4.7 \pm 0.57 \times 10^{-7}$	$2.1 - 4.0 - 5.86 - 6.23 \times 10^{-7}$ [35–38]

The excipients for the formulation of the tablets have been chosen in order to minimize osmotic stress on the Caco-2 cell monolayer. Therefore, the fraction of soluble excipients was selected to be lower than 30%. Considering the relative high volume of dissolution media and the relative low mass of the tablets, osmotic effects on the cells are highly unlikely due to dilution. Additionally, the excipients have been selected in order to prevent filter clogging (e.g. no gel formation agents). Furthermore, all used excipients yet have not been reported to affect drug permeation through Caco-2 monolayers.

4.2. Assessment of drug permeability and dissolution at different dosage strengths

The 5 and 10 mg propranolol HCl immediate release tablets, as shown in Figs. 2A–C, yielded a dissolution profile as expected for immediate release tablets. Dissolution profile of the 20 mg propranolol HCl immediate release tablet showed a minor shoulder at the beginning of the experiments (at about 10 min), whereas the dissolution profile of the 5 and 10 mg propranolol HCl can be seen as almost perfectly shaped peak. The shoulder of the 20 mg dosage form could be ascribed to the high content of propranolol HCl in the tablet with a ratio of 20% (m/m) propranolol HCl. This high amount may affect dissolution properties of the tablets or slow down disintegration of the tablet. Dissolution profiles obtained using two 10 mg propranolol HCl tablets instead of one 20 mg tablet did not show such shoulder and thus support this hypothesis (data not shown). Additionally, the saturation solubility of propranolol is reported to be approximately 100,000 μg/ml [20] and this concentration has by far never been reached. Comparing the two profiles obtained from sampling port D and A (Fig. 2A+B), one can see that the peaks measured at sampling port A were flattened compared to those measured at D, and additionally, a time shift of about 10 min had occurred. Both phenomena, flattening and time shift, are unwanted but inevitable in a flow through system. Undesired convection and mixing processes in the tubes, the stream splitter in the FTPC and time for physical transport from D to A could be seen as causes for that. For that reason, minimizing these influences may be an issue for further optimization of the apparatus since all the factors are affecting the original dissolution signal. Nevertheless, it can be pointed out that the shape and the character of the peaks did not change significantly.

The function of the stream splitter is of vital interest for the whole apparatus Therefore, linearity of the stream splitter connecting both modules and being responsible for providing a decreased stream to the permeation module was tested. As the stream splitter divided the incoming 6.5 ml/min stream into a 1.0 ml/min and a 5.5 ml stream, the ratio between mass inserted into the dissolution module and cumulative mass measured at A should be $1/6.5 = 0.1538$. Linear regression of cumulative amounts at A versus the mass of drug in the tablet (see Table 4) led to a slope of 0.154 ± 0.007 ($R^2 > 0.999$) and matches quite precisely the theoretical values, thus indicating high precision and high reproducibility of the stream splitter.

Granted that also the permeation part of the system works linear, the amount which permeated after 120 min and after 60 min through the Caco-2 monolayer should be directly proportional to the dosage strength of the inserted tablet. Therefore, the permeated amounts after 60 and 120 min were correlated with the dosage strength. As it can be seen in Table 4, the permeated amount after 60 and 120 min nicely follows the amount of drug inserted in the dissolution module, which is expected since permeation of propranolol as a passively transported compound [21] is linear. Linearity of both regressions is described by a R^2 greater than 0.999 and indicates good reproducibility and high precision of both, the permeation and dissolution measurement. The ratio between the cumulative amount appeared at A after 120 or 180 min and the respective final amount of propranolol HCl found in the basolateral compartment are shown in Table 4. These ratios are consistent with data already published in the literature, e.g. Miyazaki et al. [14] reported for the high permeable compound caffeine a ratio of 0.396%.

Table 4
Synopsis of data for the IR and the ER tablets (means ± SD, $n = 4$–5)

	Dosage strength ($n = 10$) (mg)	Amount appeared at A (mg)	Amount permeated (μg) after 60 min	Amount permeated (μg) after 120 min[a]	Ratio[b] (%)
5 mg IR tablet	5.33 ± 0.21	0.68 ± 0.03	1.98 ± 0.42	2.34 ± 0.47	0.343 ± 0.082
10 mg IR tablet	9.70 ± 0.25	1.30 ± 0.02	3.77 ± 0.50	4.23 ± 0.45	0.325 ± 0.039
20 mg IR tablet	19.23 ± 0.54	2.81 ± 0.22	6.82 ± 0.23	8.40 ± 0.32	0.292 ± 0.034
	R^2[c]	0.9998	0.9998	<0.9999	
	Slope	0.1541	0.3462	0.4365	
10 mg ER tablet 4% Eudragit® NE 30D	9.80 ± 0.35	1.35 ± 0.06		4.55 ± 0.34	0.336 ± 0.040
10 mg ER tablet 8% Eudragit® NE 30D	10.29 ± 0.18	1.16 ± 0.04		4.40 ± 0.28	0.378 ± 0.037

[a] Values given refer to different time points: for the IR tablets 120 and 180 min for the ER tablet.
[b] The ratio has been calculated as follow: cumulative amount appeared at A at the end of the experiment divided by the amount permeated through the Caco-2 monolayer (detected at B).
[c] R^2 indicates the correlation between the dosage strength and the amount appeared at the respective sampling port.

4.3. Assessment of drug permeability and dissolution at different release kinetics

As mentioned before, one can detect in Figs. 3A and B a flattening and broadening of the dissolution peaks throughout the passage of the apparatus. Again the shape of the peaks remained the same. Measuring permeation of prolonged release tablets raises the question for the maximum assay time. Small intestinal transit time is generally considered as 3.3 h [32] or is reported to range between 3.5 and 4.5 h [33]. Viability of Caco-2 cells in KRB and in other HBSS-like buffers is most probably limited to approximately 5–7 h, and consequently limitation in assay time might be irrelevant.

Considering the appearance of propranolol HCl in the basolateral compartment, a visible delay was found for the extended release tablets. After 180 min, however, permeated amount was for all different release kinetics fairly equal (Fig. 3C). For the permeation process in an open system, two variables can be considered as rate determining for permeation in this case. First, the concentration difference according to Fick's first law – the driving force for diffusion – and second, the contact time of propranolol HCl with the Caco-2 cells – the probability which is given for propranolol to permeate. Considering the total permeated amount, prolongation of release from propranolol HCl tablets is affecting both variables controversially. For that reason, the final mass of the three tablets species in the basolateral compartment, as compared in Fig. 3C, seemed to be rather equal (Table 4). As the retardation increased, slightly increasing ratios were found. Since retardation of propranolol HCl release was only moderate, the differences of the quotients were statistically not significant (one way ANOVA). Higher degrees of retardation will presumably lead to more profound effects on permeation.

In order to compare the profiles of the extended release tablets, mean times for the different sampling ports were calculated according to Eqs. (2)–(4). If MT_D is plotted against MT_A, linear regression leads to the following equation (graph not shown)

$$MT_A = 16.04 \pm 0.60 \text{ min} + 0.78 \pm 0.015 \cdot MT_D \quad R^2 > 0.999 \tag{5}$$

The intercept of approximately 16 min can be regarded as time for drug release added the time needed for physical transport from the dissolution vessel to the apical compartment of the FTPC. The targeted value for the slope is 1 and is not matched exactly; mixing and convection appeared to affect that value. High linearity ($R^2 > 0.999$) suggests high reproducibility for the transport throughout the apparatus. Fig. 4 plots both, MT_D and MT_A, versus MT_B. Regression leads to the following equations

$$MT_B = 18.01 \pm 3.50 + 0.85 \pm 0.090 \cdot MT_D \quad R^2 > 0.99 \tag{6}$$

$$MT_B = 0.62 \pm 6.28 + 1.08 \pm 0.13 \cdot MT_A \quad R^2 > 0.99 \tag{7}$$

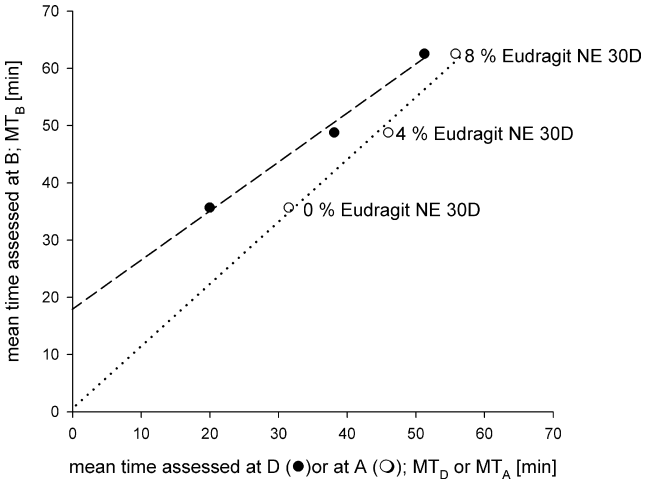

Fig. 4. Mean times of the three differently retarded tablets assessed from sampling port B are plotted (i) against mean times assessed from sampling port D (●) and (ii) against mean time assessed from sampling port A (○). $R^2 < 0.99$ for both regression lines.

The intercept of approximately 18 min in Eq. (6) can be attributed to the time of physical transport of the drug from the dissolution vessel to the apical compartment of the FTPC and subsequent permeation. The difference between the intercept of Eqs. (5) and (6) of approximately 2 min can roughly be estimated as time of permeation. The slope of 0.85 is close to the targeted value of 1; again, mixing and convection might have affected the signal. The intercept of Eq. (7) was very small, which was expected. Physical transport can be considered as negligible, since, due to equal tubing, transport from the FTPC to both sampling ports happened within the same time. Here, the calculated slope matched precisely the targeted value of 1. For confirmation, mean time for permeation was calculated according to the following equation

mean time for permeation :

$$MP = P_{app} \cdot \text{monolayer thickness} \tag{8}$$

Assuming a Caco-2 monolayer height of approximately 20–30 μm [34] and a P_{app} of 2.5 10^{-5} cm/s, MP can roughly be estimated as 80–120 s. This value apparently corresponds well with the intercept of Eq. (7) and the difference of the intercepts of Eq. (6) to (5). In summary, it can be pointed out that using a mean time approach consistent and plausible explanation for the different concentration time profiles were found.

In contrast to previously published setup, the here described apparatus facilitates the use of complete oral dosage forms with physiologically relevant volumes and drug concentrations. Beyond that, the use of a commonly accepted and extensively described compendial dissolution device entails the advantage of comparability and reliability. A further advantage is obviously the installation of the stream splitter dividing the dissolution and permeation system into two hydrodynamically independent subunits.

This facilitates, in comparison to previous setup, the application of pumps in the dissolution module as they are generally recommended for flow through dissolution cells. Thorough drug quantification at various sampling port facilitates a better tracing of drug in the apparatus. Nevertheless, the here described apparatus is lacking a unit mimicking the gastrointestinal pH gradient. Additionally, efforts for performing experiments have been increased.

After all, it is worth to mention that is was not intended to mimic perfectly *in vivo* dissolution and permeation conditions. Although, it was possible to simulate the estimated volumes of liquid accessible for dissolution and intestinal concentrations, it is up to now not possible to simulate the area accessible for permeation and present *in vivo*, which would be deducive for permeation when mimicking the volumes in the gastrointestinal tract. It remains, however, unclear whether results from such perfect *in vivo* apparatus will achieve adequate results when comparing to the financial efforts which have to be made therefore.

Summarizing these experimental results, one can conclude that our new apparatus was able to monitor permeation through a Caco-2 monolayer of drugs formulated as a solid dosage form. The apparatus showed linear behaviour for increasing dosage strengths of propranolol HCl immediate release tablets and also linear behaviour in terms of retardation of release.

5. Conclusion

A successful installation of a promising novel apparatus for the assessment of intestinal permeability of drugs formulated as solid oral dosage forms was realized by combining a flow through Caco-2 permeation cell with a compendial flow though dissolution cell (USP apparatus 4). This combination of established, but so far only separately used, research tools yields plausible data, not only for immediate but also for extended release tablets of the BCS class I compound propranolol HCl. Problems arising from applying compendial flow through dissolution devices were adequately solved by a stream splitter. The above shown data provide the basis for more advanced studies on the influence of dosage form, excipients and other factors on the permeation as well as the dissolution process.

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References

[1] P. Artursson, J. Karlsson, Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells, Biochem. Biophys. Res. Commun 175 (1991) 880–885.
[2] P. Artursson, K. Palm, K. Luthman, Caco-2 monolayers in experimental and theoretical predictions of drug transport, Adv. Drug. Del. Rev 22 (1996) 67–84.
[3] P. Artursson, K. Palm, K. Luthman, Caco-2 monolayers in experimental and theoretical predictions of drug transport, Adv. Drug. Del. Rev. 46 (2001) 27–43.
[4] J. Raiman, S. Tormalehto, K. Yritys, H.E. Junginger, J. Monkkonen, Effects of various absorption enhancers on transport of clodronate through Caco-2 cells, Int. J. Pharm. 261 (2003) 129–136.
[5] M. Thanou, J.C. Verhoef, H.E. Junginger, Oral drug absorption enhancement by chitosan and its derivatives, Adv. Drug. Del. Rev. 52 (2001) 117–126.
[6] K. Bogman, Y. Zysset, L. Degen, G. Hopfgartner, H. Gutmann, J. Alsenz, J. Drewe, P-glycoprotein and surfactants: effect on intestinal talinolol absorption, Clin. Pharmacol. Ther. 77 (2005) 24–32.
[7] E.D. Hugger, B.L. Novak, P.S. Burton, K.L. Audus, R.T. Borchardt, A comparison of commonly used polyethoxylated pharmaceutical excipients on their ability to inhibit P-glycoprotein activity *in vitro*, J. Pharm. Sci. 91 (2002) 1991–2002.
[8] B.D. Rege, J.P. Kao, J.E. Polli, Effects of nonionic surfactants on membrane transporters in Caco-2 cell monolayers, Eur. J. Pharm. Sci. 16 (2002) 237–246.
[9] B.D. Rege, L.X. Yu, A.S. Hussain, J.E. Polli, Effect of common excipients on Caco-2 transport of low-permeability drugs, J. Pharm. Sci. 90 (2001) 1776–1786.
[10] D. Wagner, H. Spahn-Langguth, A. Hanafy, A. Koggel, P. Langguth, Intestinal drug efflux: formulation and food effects, Adv. Drug. Del. Rev. 50 (Suppl. 1) (2001) S13–S31, Pflügers Archiv European Journal of Physiology.
[11] J. Brouwers, J. Tack, F. Lammert, P. Augustijns, Intraluminal drug and formulation behavior and integration *in vitro* permeability estimation: a case study with amprenavir, J. Pharm. Sci. 95 (2006) 372–383.
[12] M.J. Ginski, R. Taneja, J.E. Polli, Prediction of dissolution-absorption relationships from a continuous dissolution/Caco-2 system, AAPS PharmSci. 1 (1999) E3.
[13] M. Kataoka, Y. Masaoka, Y. Yamazaki, T. Sakane, H. Sezaki, S. Yamashita, *In vitro* system to evaluate oral absorption of poorly water-soluble drugs: simultaneous analysis on dissolution and permeation of drugs, Pharm. Res. 20 (2003) 1674–1680.
[14] X. He, M. Sugawara, M. Kobayashi, Y. Takekuma, K. Miyazaki, An *in vitro* system for prediction of oral absorption of relatively water-soluble drugs and ester prodrugs, Int. J. Pharm. 263 (2003) 35–44.
[15] X. He, S. Kadomura, Y. Takekuma, M. Sugawara, K. Miyazaki, A new system for the prediction of drug absorption using a pH-controlled Caco-2 model: evaluation of pH-dependent soluble drug absorption and pH-related changes in absorption, J. Pharm. Sci. 93 (2004) 71–77.
[16] M. Sugawara, Y. Takekuma, K. Miyazaki, S. Kadomura, X. He, N. Kohri, The use of an *in vitro* dissolution and absorption system to evaluate oral absorption of two weak bases in pH-independent controlled-release formulations, Eur. J. Pharm. Sci. 26 (2005) 1–8.
[17] M. Kobayashi, N. Sada, M. Sugawara, K. Iseki, K. Miyazaki, Development of a new system for prediction of drug absorption that takes into account drug dissolution and pH change in the gastrointestinal tract, Int. J. Pharm. 221 (2001) 87–94.
[18] H. Vogelpoel, J. Welink, G.L. Amidon, H.E. Junginger, K.K. Midha, H. Moller, M. Olling, V.P. Shah, D.M. Barends, Biowaiver monographs for immediate release solid oral dosage forms based on biopharmaceutics classification system (BCS) literature data: verapamil hydrochloride, propranolol hydrochloride, and atenolol, J. Pharm. Sci. 93 (2004) 1945–1956.

[19] N. Takamatsu, L.S. Welage, N.M. Idkaidek, D.-Y. Liu, P.I.-D. Lee, Y. Hayashi, J.K. Rhie, H. Lennernas, J.L. Barnett, V.P. Shah, L. Lesko, G.L. Amidon, Human intestinal permeability of piroxicam, propranolol, phenylalanine, and PEG 400 determined by jejunal perfusion, Pharm. Res. 14 (1997) 1127–1132.
[20] V. Pade, S. Stavchansky, Link between drug absorption solubility and permeability measurements in Caco-2 cells, J. Pharm. Sci. 87 (1998) 1604–1607.
[21] V. Pade, S. Stavchansky, Estimation of the relative contribution of the transcellular and paracellular pathway to the transport of passively absorbed drugs in the Caco-2 cell culture model, Pharm. Res. 14 (1997) 1210–1215.
[22] S. Takka, S. Rajbhandari, A. Sakr, Effect of anionic polymers on the release of propranolol hydrochloride from matrix tablets, Eur. J. Pharm. Biopharm. 52 (2001) 75–82.
[23] C.-Y. Wu, L.Z. Benet, Predicting drug disposition via application of BCS: transport/absorption/elimination interplay and development of a biopharmaceutics drug disposition classification system, Pharm. Res. 22 (2005) 11–23.
[24] M. Lindenberg, S. Kopp, J.B. Dressman, Classification of orally administered drugs on the World Health Organization Model list of Essential Medicines according to the biopharmaceutics classification system, Eur. J. Pharm. Biopharm. 58 (2004) 265–278.
[25] USP. The United States Pharmacopoeia/The National Formulary. United States Pharmacopoeial Convention Inc., Rockville, USA, 2004.
[26] F. Ingels, S. Deferme, E. Destexhe, M. Oth, G. Van den Mooter, P. Augustijns, Simulated intestinal fluid as transport medium in the Caco-2 cell culture model, Int. J. Pharm. 232 (2002) 183–192.
[27] S. Neuhoff, A.L. Ungell, I. Zamora, P. Artursson, pH-dependent bidirectional transport of weakly basic drugs across Caco-2 monolayers: implications for drug–drug interactions, Pharm. Res. 20 (2003) 1141–1148.
[28] R.L. Dillard, H. Eastman, J.S. Fordtran, Volume-flow relationship during the transport of fluid through the human small intestine, Gastroenterology 49 (1965) 58–66.
[29] J.S. Fordtran, T.W. Locklear, Ionic constituents and osmolality of gastric and small-intestinal fluids after eating, Am. J. Dig. Dis. 11 (1966) 503–521.
[30] V.H. Sunesen, B.L. Pedersen, H.G. Kristensen, A. Müllertz, *In vivo in vitro* correlations for a poorly soluble drug, danazol, using the flow-through dissolution method with biorelevant dissolution media, Eur. J. Pharm. Sci. 24 (2005) 305–313.
[31] J. Karlsson, P. Artursson, A method for the determination of cellular permeability coefficients and aqueous boundary layer thickness in monolayers of intestinal epithelial (Caco-2) cells grown in permeable filter chambers, Int. J. Pharm. 71 (1991) 55–64.
[32] L.X. Yu, G.L. Amidon, J.R. Crison, Compartmental transit and dispersion model analysis of small intestinal transit flow in humans, Int. J. Pharm. 140 (1996) 111–118.
[33] J. Dressman, J. Krämer, Pharmaceutical Dissolution Testing, Taylor & Francis Group, Boca Raton, USA, 2005.
[34] M.D. Peterson, M.S. Mooseker, An *in vitro* model for the analysis of intestinal brush border assembly. I. Ultrastructural analysis of cell contact-induced brush border assembly in Caco-2(BBe) cells, J. Cell Sci. 105 (1993) 445–460.
[35] E. Duizer, A.H. Penninks, W.H. Stenhuis, J.P. Groten, Comparison of permeability characteristics of the human colonic Caco-2 and rat small intestinal IEC-18 cell lines, J. Controlled Release 49 (1997) 39–49.
[36] T. Lindmark, Y. Kimura, P. Artursson, Absorption enhancement through intracellular regulation of tight junction permeability by medium chain fatty acids in Caco-2 cells, J. Pharmacol. Exp. Ther. 284 (1998) 362–369.
[37] M. Sakai, T. Imai, H. Ohtake, H. Azuma, M. Otagiri, Effects of absorption enhancers on the transport of model compounds in Caco-2 cell monolayers: assessment by confocal laser scanning microscopy, J. Pharm. Sci. 86 (1997) 779–785.
[38] T. Imai, M. Sakai, H. Ohtake, H. Azuma, M. Otagiri, *In vitro* and *in vivo* evaluation of the enhancing activity of glycyrrhizin on the intestinal absorption of drugs, Pharm. Res. 16 (1999) 80–86.