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Research paper

Evaluation of cocktail approach to standardise Caco-2 permeability experiments

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Abstract

The purpose of this study was to investigate the suitability and reliability of *n*-in-one approach using FDA suggested compounds for standardising Caco-2 permeability experiments. Special attention was paid to the evaluation of rank order correlation and mechanistic insights of compound permeability. Transport studies with antipyrine, metoprolol, ketoprofen, verapamil, hydrochlorothiazide, ranitidine, mannitol and fluorescein were performed in 12- and 24-well formats, as single compounds and in cocktails under iso-pH 7.4 and pH-gradient (pH 5.5 vs. 7.4) conditions. Compounds were quantified using *n*-in-one LC/MS/MS analysis. The cocktail-dosing proved to be a feasible method to determine the permeability of the Caco-2 cell line and to introduce external standards for permeability tests. Even though sink conditions were lost in cocktail experiments for highly permeable compounds, the rank order of compound permeability and the classification to low and high permeability compounds remained unchanged between single and cocktail studies and permeability values of 12- and 24-well formats were directly comparable. Under pH-gradient conditions the margin between high and low permeability compounds was narrower due to the lower permeability (higher fraction of ionisation) of basic molecules. Of the compounds studied, antipyrine, metoprolol, hydrochlorothiazide and mannitol are suitable for evaluation and standardisation purposes of passive permeability, while fluorescein would function as paracellular marker under iso-pH 7.4. As efflux activity may vary between cell batches, verapamil is a useful marker for *P*-glycoprotein.

Keywords: Caco-2 monolayers; *n*-in-one cocktail; Standardisation; Drug permeability; Transport mechanism

1. Introduction

Authorities have approved in vitro permeation studies across the cultured epithelial cells for appraising permeability properties of compounds for immediate-release solid oral dosage forms [1]. This guidance is based on a biopharmaceutics classification system (BCS) of drugs according to their solubility and permeability behaviour [2]. Caco-2 cells are widely used as an in vitro method for drug permeability determination, because these cells have similar characteristics as the absorptive cells in the human intestine [3,4]. In addition to BCS classification, Caco-2 cells can be used as a screening method for new drug candidates at early stages of the drug discovery process. For any predictive purposes, the suitability and reliability of the permeability method must be demonstrated using a range of model drugs with a known intestinal absorption in humans. These model drugs should represent a range of low (<50%), moderate (50–89%) and high (⩾90%) fraction absorbed (fa%).

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FDA recommends 20 model drugs for this method validation, the compounds being listed as having high, low and zero permeability, and one also as a potential marker for *P*-glycoprotein efflux activity (verapamil). Some of these model compounds (antipyrine, metoprolol and mannitol) are suggested as potential internal standards, the prerequisite for internals standards being that there are no interactions with co-administered compounds.

Combinatorial synthesis produces a lot of compounds, which in turn creates the pressure for rapid early ADME screening. In order to increase throughput and to limit consumption of (newly synthesized) drug candidates, sample pooling for analysis or permeation studies using cocktails of model drugs (mixture dosing, cassette dosing) and utilisation of smaller scale inserts have been investigated in recent years [5–13]. Analysis of several compounds over a large concentration range from the individual samples requires sophisticated and sensitive analytical methodology [9]. Unless high concentrations of poorly permeable compounds are used, the sensitivity of the analytical method for the compounds of lowest permeability determines also the sampling interval, which may result in underestimation of permeability of highly permeable drugs due to the loss of sink conditions.

Standardisation of Caco-2 cell model with reference compounds is essential to ensure the validity of the permeability results of the compounds under investigation [14–16]. In addition to the well-known variability in permeability values between laboratories [4], the variability within a laboratory must also be recognised [17]. Therefore the culturing protocol, filter supports, cell passage numbers used in the experiments and experimental conditions (pH, stirring, and drug concentration) should be carefully defined. Thus, the routine check of model compound permeabilities and the use of internal standards and marker molecules in permeability experiments are necessary. The expression and function of active transporters are also known to vary between the Caco-2 cell passages and batches [18]. Therefore, the knowledge of model substrate behaviour in Caco-2 experiments is essential when evaluating the factors potentially contributing to the permeability mechanism of drug candidates.

The purpose of this study was to investigate the suitability and reliability of an *n*-in-one permeability approach for standardising Caco-2 permeability experiments, utilising *n*-in-one LC/MS/MS methodology developed for the analytics [9]. Special attention was paid to the method applicability, evaluation of rank order correlation and mechanistic insights of compound permeability. Permeability experiments with a selection of FDA suggested compounds were conducted in single-compound and cocktail systems utilising 12- and 24-well formats under iso-pH 7.4 and pH-gradient conditions. The compounds were chosen to represent different structures, permeation mechanisms and physico-chemical characteristics (pK_a, lipophilicity), and could be designated to three of the four BCS classes: Antipyrine and metoprolol belong to class 1 (high solubility and permeability), verapamil and ketoprofen to class 2 (low solubility, high permeability), and ranitidine, hydrochlorothiazide and mannitol to class 3 (high solubility, low permeability) [19] (Table 1). Several of the compounds permeate mainly through passive transcellular (antipyrine, metoprolol, ketoprofen) [20] and/or paracellular (ranitidine, hydrochlorothiazide, mannitol, fluorescein) diffusion, especially under iso-pH conditions. However, involvement of efflux mechanisms for ranitidine [21,22] and active transport for ketoprofen [10,23] and fluorescein [24,25] under pH-gradient conditions have been suggested. Verapamil is a known substrate of the *P*-glycoprotein (MDR1) efflux protein and as such is suggested as marker compound for *P*-glycoprotein, while displaying also high passive permeability [26]. Radioactive mannitol was used as a cell membrane integrity marker after the permeation experiments. The feasibility of fluorescein as an alternative for mannitol as internal standard of paracellular permeability was studied in order to obtain more rapid information of monolayer integrity.

2. Materials and methods

2.1. Compounds

Verapamil hydrochloride, ketoprofen and metoprolol tartrate were purchased from ICN Biomedicals Inc. (Aurora, OH, USA), antipyrine was from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA) and ranitidine hydrochloride, fluorescein sodium and hydrochlorothiazide were from Sigma–Aldrich Chemie (Steinheim, Germany). D-[1-^{14}C]-mannitol (specific activity 59.0 mCi/mmol) was purchased from Amersham Pharmacia Biotech (England).

2.2. Cell culture

Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD). The growth media Dulbecco's modified Eagle's medium (DMEM), nonessential amino acids (NEAA), L-glutamine (200 mM), penicillin G (10,000 IU/ml)/streptomycin sulphate (10,000 μg/ml) solution, Dulbecco's phosphate-buffered saline (PBS) and Trypsin solution (2.5%) were purchased from Euroclone Ltd. (England). Hepes [4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid] and MES [2-(*N*-morpholino)-ethanesulphonic acid were from Sigma–Aldrich Chemie (Steinheim, Germany). The foetal calf serum (FBS) and the transport medium HBSS (Hanks' balanced salt solution) were from Gibco Invitrogen Corp. (Life Technologies Lts., Paisley, Scotland).

Caco-2 cells were grown in tissue culture flasks (Corning Costar Corp., Cambridge, MA) (75 cm^2) in DMEM supplemented with 10% FBS (heat inactivated), 1% NEAA, 2 mM L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin sulphate. The growth medium was changed every 2–3 days and sub-cultured at 80% confluence by trypsinisation. Cells at passages 32–42 were seeded on

Table 1
Characteristics of the model compounds in the cocktails

Compound	Fa%	Permeability class according to FDA/transport mechanism	pK_a	Log P	Log $D_{7.4}$	Log $D_{5.5}$
Antipyrine	100 [44], 97 [45]	High (potential internal standard)/passive transcellular	1.44 [47]	0.56 [47]	0.6 [47]	0.6 [47]
Ketoprofen	100 [44]	High/active transport	3.89 [47]	3.37 [47]	0.1 [47]	1.8 [47]
Metoprolol	95 [44]	High (potential internal standard)/passive transcellular	9.60 [47]	2.07 [47]	0.0 [47]	−0.6 [47]
Verapamil	98 [44], 100 [45]	High/efflux pump substrate candidate	8.66 [47]	3.96 [47]	2.7 [47]	1.2 [47]
Ranitidine	55 [44], 50 [45]	Low (moderate)/passive paracellular, facilitated diffusion	8.66 [48]	1.03 [48]	−0.25[a]	−2.13[a]
Hydrochloro-thiazide	70 [44], 67 [45]	Low (moderate)/passive paracellular	8.84 [47] 10.07	−0.17 [47]	−0.2 [47]	−0.2 [47]
Mannitol	16 [44], 26 [46]	Low (potential internal standard)/passive paracellular				
Fluorescein		Potential paracellular marker at pH 7.4/active transport with pH-gradient	4.29[b], 6.37	3.59[b]	−0.36[b]	2.31[b]

Fa%, fraction absorbed in humans.
[a] Log D calculated based on pK_a and log P values.
[b] Determined in our laboratory. See for details in Ref. [49].

polycarbonate Transwell® inserts (Corning Costar Corp., Cambridge, MA, pore size 0.4 μm, growth area 1.1 cm^2 or 0.33 cm^2) at a density of 68,000 cells/cm^2. The monolayers were grown for 22–27 days before the transport experiments. The culturing conditions were 37 °C in an atmosphere of 90% relative humidity and 5% CO_2.

2.3. Permeability experiments

The experiments were performed in the apical to basolateral (AP–BL) and basolateral to apical (BL–AP) directions under non-gradient pH conditions (pH 7.4 on both sides; iso-pH 7.4) and pH-gradient conditions (pH 5.5 apically, pH 7.4 basolaterally). The permeability of each compound was studied in 12- and 24-well formats (inserts with areas of 1.1 and 0.33 cm^2) as a single compound and in a cocktail. HBSS containing 10 mM Hepes (pH adjusted to 7.4) or MES (pH adjusted to 5.5) was used as transport medium. Fully differentiated cell monolayers were washed twice with pre-warmed transport medium (HBSS/Hepes) and equilibrated for 30 min in HBSS (pH 7.4/5.5 apically and pH 7.4 basolaterally). The drug solutions were prepared in HBSS, pH 7.4 or 5.5, at 50 μM concentrations. The AP–BL permeability experiment was started by adding 0.4 ml (1.1 cm^2 monolayers) or 0.15 ml (0.33 cm^2 monolayers) of pre-warmed drug solutions containing the compound(s) of interest on the apical side. The basolateral side contained 1.6 or 0.6 ml of fresh HBSS, respectively, in order to keep the volume ratios of the apical and basolateral sides constant for both the insert sizes. Cells were kept in an orbital shaker incubator (Stuart, Bibby Sterilin Ltd.) at 37 °C at a constant stirring rate (50 rev/min) during the experiments. The samples were taken at 2–30 min time intervals by transferring the cell inserts into new wells containing fresh HBSS. The BL–AP permeability experiment was started by adding 1.6 or 0.6 ml of drug solution on the basolateral side. The samples of 0.3 or 0.1 ml were taken from the apical side and the volume was replaced with fresh HBSS.

For the single-compound experiments the sampling intervals were chosen so that sink-conditions would apply (i.e. the overall transported amount would not exceed 10% of the amount in the donor solution). For the cocktails, the sampling intervals were 30 and 15 min for AP–BL and BL–AP directions, respectively. Compound recovery was assessed by calculating the sum of the cumulatively transported amount and the amount left in the donor side against the initial amount of drug. The samples were stored at −70 °C until analysis. Prior to experiments, the integrity of the monolayers was ensured by TEER (transepithelial electrical resistance)-measurements and monolayers exhibiting TEER-values under 200 Ω cm^2 were discarded. In addition to TEER-measurements, the permeability of radiolabelled mannitol (paracellular marker molecule) was measured after the experiments. Mannitol solutions were added on the apical side and incubated for 60 min. The samples of 100 μl were analysed by liquid

scintillation counter (Wallac, Turku, Finland) using 4 ml of HiSafe 2 (Wallac Scintillation Products, Fisher Chemicals, Loughborough, UK) as a scintillation cocktail. The transport rate should not exceed the limit value of 0.5%/h.

The flux (J) of the drug was determined from the slope of the plot of the cumulatively transported amount (nmol) versus time (min). The apparent permeability coefficients (Papp, cm/s) were calculated according to the equation:

$$\text{Papp} = \frac{(\mathrm{d}Q/\mathrm{d}t)}{A \times C_0 \times 60}, \quad (1)$$

where $\mathrm{d}Q/\mathrm{d}t$ is the amount of compound transported within a given time period (nmol/min), A is the surface area of the insert (cm^2) and C_0 is the initial drug concentration (nmol/ml).

Permeability experiments were performed in triplicate across all studied compounds and their mixtures. Some experiments were repeated with a second batch of cells in order to assess the sensitivity of verapamil as a marker for *P*-glycoprotein activity and in order to resolve some issues regarding assay sensitivity and sample pooling.

2.4. Analytical procedures

The analytical method, liquid chromatography/tandem mass spectrometry (LC/MS/MS) using atmospheric pressure photoionisation (APPI) (Machine Shop, University of Groeningen, The Netherlands), was originally developed for ten compounds, of which six were used in this study [9]. Here, an Agilent HP 1100 HPLC-system equipped with a column switching option (Hewlett–Packard GmbH, Waldbronn, Germany) and a Sciex API 3000 mass spectrometer (Applied Biosystems, Toronto, Canada) was used. Some assays were carried out using turbo ionspray (Applied Biosystems, Toronto, Canada) according to the method described earlier [9]. Stock mixture containing each of the six compounds at 10 mM in methanol or acetonitrile was prepared and then further diluted with HBSS (pH 7.4 and 5.5) to the appropriate concentrations for calibration curves (10–5000 nM). Permeability samples were centrifuged for 3 min at 3000 rpm by Eppendorf centrifuge 5451 D (Eppendorf AG, Hamburg, Germany) prior to pipetting into 96-well plates and injection into the LC/MS/MS system. A reversed-phase monolithic column, Chromolith SpeedROD, RP-18e, 4.6 × 50 mm (Merck, Darmstadt, Germany), was operated at 2 ml/min using an injection volume of 40 μl. The mobile phase system consisted of 10 mM ammonium acetate without pH adjustment (A) and acetonitrile (B). A linear gradient elution profile was used as follows: 10% B for 0–0.8 min, 10–90% B for 0.8–2 min, 90% B for 2–3.5 min, 90–10% B for 3.5–4 min and finally 1.5 min equilibration before the injection of the next sample. A column switching system was used to direct the early eluting salts, i.e. the first 0.8 min of flow, to waste. Antipyrine, ketoprofen, metoprolol and verapamil were detected using positive and ranitidine and hydrochlorothiazide using negative ion multiple reaction monitoring. During the first 1.7 min data were collected in negative ion mode after which the polarity was changed to positive ion mode. The mounting bracket voltage in the positive and negative ion modes was +1300 and −1300 V, respectively. Declustering potential, collision energy and collision cell exit potential were individually optimised for each compound [9]. The dwell time was 70 ms for each monitored ion pair during the positive mode and 170 ms during the negative mode period. HPLC-grade toluene was used as a dopant at a flow rate of 20 μl/min. Compressed air (Atlas Copco air-dryer, Wilrijk, Belgium) at 1.2 l/min was used as an auxiliary gas. High-purity nitrogen (99.999%, Oy Woikoski Ab, Voikoski, Finland) at 1 l/min was used as a lamp gas and nitrogen produced by a nitrogen generator (Whatman 75–720) was used as a curtain and nebulizer gas. Flow rate of the curtain gas was 1.2 l/min. Pressure of the nebulizer was 75 bar and temperature 425 °C. The data were collected using a Dell OPTIPLEX computer and processed with PE Sciex Analyst 1.2 software.

Fluorescein was analysed using a fluorescent plate reader (Victor 1420, Wallac Oy, Turku, Finland) at 485 and 535 nm as the excitation and emission wavelengths. Due to a higher absorption and emission in fully ionised form, the pH of the samples was adjusted to pH 9 before the analysis. The calibration range for fluorescein was 0.005–60 μM.

3. Results and discussion

3.1. Utility of the cocktail approach

The rank order of apical to basolateral compound transport and classification of low and high permeability compounds were a perfect match between the single and cocktail studies at iso-pH 7.4, even though the sink conditions did not apply for the highly permeable compounds like antipyrine, ketoprofen and metoprolol in the cocktail experiments (Table 2). Transport experiments of single compounds were performed under sink-conditions, and the sampling intervals varied from 2 to 30 min for the compounds of high and low permeability, respectively. With the cocktails, the sampling interval was selected according to the lowest permeable compound and the sensitivity of the analytics (30 min in the AP–BL and 15 min in the BL–AP direction). The apparent permeability coefficients of highly permeable compounds were lower in cocktails due to the long sampling interval producing non-sink conditions, which is also likely to allow back-diffusion. The application of Eq. (1) for Papp calculation is, thus, not entirely valid for the non-sink conditions. The fraction of permeated amount of antipyrine for example was about 20% in 30 min. Thereupon the cut-off value for highly permeable compounds under iso-pH was lowered from 30×10^{-6} cm/s (single) to 20×10^{-6} cm/s (cocktail) (Table 2). In order to use shorter sampling intervals, the initial individual drug concentrations of poorly permeable

Table 2
Apparent permeability coefficients (Papp $\times 10^{-6}$ cm/s) of the model compounds as single compounds and in cocktails in AP–BL direction under iso-pH (pH 7.4 on both sides) pH-gradient conditions (pH 5.5 on apical and pH 7.4 on basolateral) across 1.1 cm^2 Caco-2 monolayers (means ± SD, $n = 3$)

Compound	Iso-pH 7.4		pH-gradient	
	Single	Cocktail	Single	Cocktail
Antipyrine	46.6 ± 5.3	27.1 ± 1.8	46.0 ± 5.4	27.2 ± 0.6
Metoprolol	33.4 ± 6.2	27.1 ± 0.9	1.55 ± 0.24	1.92 ± 0.13
Ketoprofen	32.3 ± 1.1	21.2 ± 1.5	79.9 ± 20.1	30.9 ± 1.9
Verapamil	2.35 ± 1.29	6.77 ± 0.17	<0.12	<0.12
Ranitidine	1.31 ± 0.20	1.16 ± 0.05	0.33 ± 0.04	0.17 ± 0.01
Hydrochlorothiazide	0.73 ± 0.09	0.36 ± 0.02	0.76 ± 0.03	0.32 ± 0.04
Fluorescein	0.36 ± 0.01	0.33 ± 0.06	28.4 ± 4.7	Not determined

compounds could be increased, but this would also increase the risk for cell damage/toxicity.

Under pH-gradient conditions the rank order was changed due to the changes in the ionisation state of ionisable molecules (Table 2). Accordingly, as the fraction of ionised form for the weak acids, ketoprofen and fluorescein, is higher at pH 7.4 than at pH 5.5, significant differences in their permeability were to be expected, partly also due to the changes in sink-conditions. The basic compounds (metoprolol, verapamil, ranitidine) display opposite ionisation behaviour (lower log D values at pH 5.5 than at 7.4; Table 2), but should similarly display pH-dependent permeability [27]. In addition, the pH-gradient acts as a driving force for some of the active transporters utilising a proton-cotransport mechanism, affecting the transport of ketoprofen and fluorescein [10,23–25,28,29]. Due to the changes in ionisation and permeability, the difference between the low and high permeability compounds was narrower and the limiting Papp value classifying the compounds to low and high permeability classes was lower with the pH-gradient (1×10^{-6} cm/s) compared to the Papp value without the pH-gradient (20×10^{-6} cm/s).

The rank order between low and moderate permeability compounds without the pH-gradient was not as clear. The transport of ranitidine across Caco-2 cell monolayers was slightly higher than that of hydrochlorothiazide, while the fraction absorbed data would suggest the opposite (Table 1). Under the pH-gradient conditions, the changeover in rank order of ranitidine and hydrochlorothiazide permeation in relation to absorption in humans were corrected. It has been suggested that the permeation across the Caco-2 cells under the pH-gradient (acidic apical pH) correlates better to the absorption in humans [30]. This is reasonable, because the transport studies of drugs under pH-gradient conditions resemble more closely the intestinal conditions during in vivo drug absorption in humans (acidic microclimate above the enterocytes in the upper part of the intestine [31]).

Good correlation between single and cocktail studies and the success in classification of the compounds to high and low permeability drugs proved that these compounds can be used as a validation tool for Caco-2 permeability. However, it is not necessary or even appropriate to use all these model drugs in one cocktail or as internal standards due to the possible interactions. Depending on the experimental setups, different information of Caco-2 cell permeability can be achieved with different compounds. Putative role of each compound and their combinations are discussed in Sections 3.2 and 3.3.

3.2. High permeability compounds

Of the highly permeable compounds, antipyrine showed consistent Papp values across all the experimental setups (single/cocktail, pH-conditions, insert sizes). The Papp values of antipyrine were between 46.0 and 58.1×10^{-6} cm/s in single studies and between 27.1 and 35.6×10^{-6} cm/s in cocktail (non-sink conditions) studies in AP–BL direction with individual coefficients of variance of replicates below 15%. Antipyrine is a suitable model compound to represent highly permeable compounds using passive transcellular route for absorption as a part of the cocktail or as an internal standard, regardless of the pH conditions used.

Metoprolol is also a good marker of passive transcellular transport and can be included in the cocktail, but the changing and polarised permeability according to the pH (Fig. 1) must be recognised and determined. The permeation of metoprolol in the AP–BL direction decreased from $33.4 \pm 6.24 \times 10^{-6}$ cm/s (iso-pH) to $1.55 \pm 0.24 \times 10^{-6}$ cm/s (pH-gradient) reducing the cut-off value of highly permeable compounds considerably. In the BL–AP direction, transport was increased from $46.1 \pm 0.5 \times 10^{-6}$ to $71.2 \pm 11.1 \times 10^{-6}$ cm/s. Hence, the efflux ratio increased remarkably under pH-gradient conditions (from 1.4 at iso-pH 7.4 to 50). This kind of behaviour is easily misinterpreted as an active efflux [27]. However, the efflux ratio of 1.4 under iso-pH conditions indicated that metoprolol is a passively permeating compound [32]. Thus, a pH-gradient may not be the best choice for studies of active efflux mechanism for basic molecules or the compounds should be studied under both iso-pH and pH-gradient conditions to obtain meaningful mechanistic information.

The distortion of the Papp values due to the non-sink conditions in cocktails with ketoprofen became even more pronounced with the pH-gradient, because about 40% of the initial amount was transported during the first 30 min. Thus, the clearly higher permeation of ketoprofen under pH-gradient conditions was not evident in cocktails

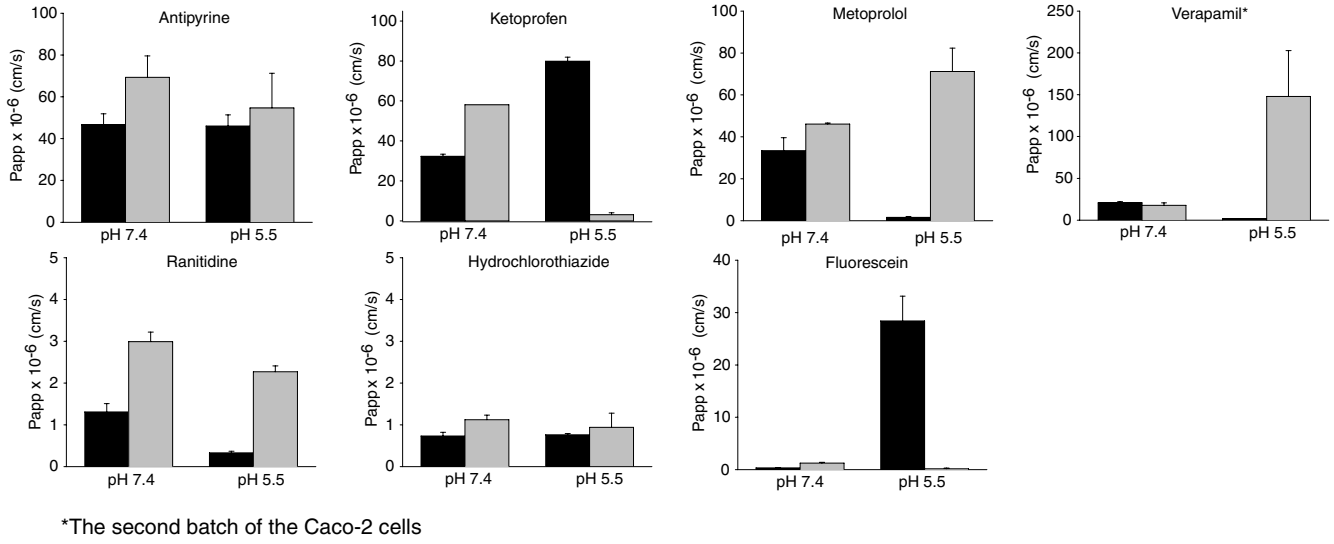

Fig. 1. Papp values of apical to basolateral (black bars) and basolateral to apical (gray bars) transport of the model compounds under pH-gradient (apical pH 5.5) and non-gradient (apical pH 7.4) conditions. The data are means ± SD, $n = 3$.

due to the application of Eq. (1) for Papp calculations (Table 1). The efflux ratio of ketoprofen changed in an opposite direction (from 1.8 at iso-pH 7.4 to 0.04 with pH-gradient) (Fig. 1) than with metoprolol when the apical pH of 5.5 was applied. Ketoprofen is a good model drug for highly permeable compounds, but unsuitable as internal standard due to its ambiguous transport mechanism (potential substrate of MCT transporters), which may introduce interactions with other monocarboxylic acids (like naproxen) [10,23,33].

Like ketoprofen, fluorescein is also reported to be a substrate of an anion transporter in its monoanionic form, although the involvement of MCTs is unclear [25]. Possible inhibitory effects of fluorescein on ketoprofen transport were not evident in our experiments and transport of ketoprofen investigated alone and with fluorescein under pH-gradient conditions gave comparable Papp values for ketoprofen ($119 \pm 12 \times 10^{-6}$ cm/s vs. $132 \pm 19 \times 10^{-6}$ cm/s). Interestingly, the transport of fluorescein was decreased from $17.1 \pm 1.76 \times 10^{-6}$ to $6.07 \pm 1.13 \times 10^{-6}$ cm/s in the presence of ketoprofen, indicating a possible interaction. Thus, the use of ketoprofen (and/or fluorescein) as internal standard(s) with a pH-gradient is risky in the presence of other anionic compounds. However, ketoprofen could possibly be utilised as an external marker for active transport.

Verapamil is also classified as a high permeability drug. The presently obtained absorptive Caco-2 cell permeability would, as such, underestimate the absorption potential of verapamil compared to humans, which is around 100% (Tables 1, 2). Permeation of verapamil (a *P*-glycoprotein substrate) was low (under the detection limit with pH-gradient) in the AP–BL direction and the variability between the replicates was high in single compound experiments (Table 2). However, the higher (>10 times) BL–AP transport of verapamil under non-gradient conditions indicated clearly the involvement of *P*-glycoprotein, as compounds with efflux ratios higher than 2 have been classified as *P*-glycoprotein substrates (at iso-pH 7.4) [32].

As it is suggested that culturing conditions and the time in culture strongly affect the expression level of *P*-glycoprotein [18], the expression and functionality of efflux protein in Caco-2 in the used passage window is being investigated in our laboratory. In the first experiments with verapamil, cells of passage numbers 36 and 40 were used. This is exactly the passage window, wherein expression (based on RT-PCR) and functionality of *P*-glycoprotein efflux action has been observed while at higher passages it appears to diminish [34,35]. In order to reach more conclusive results, the verapamil experiment was repeated with a batch of Caco-2 cells of higher passage (P42) giving now clearly higher AP–BL Papp values of $20.7 \pm 1.52 \times 10^{-6}$ cm/s (iso-pH) and $1.81 \pm 0.128 \times 10^{-6}$ cm/s (pH-gradient) (Fig. 1). Thus, under pH-gradient conditions the BL–AP/AP–BL ratio was 83, while at iso-pH 7.4 it was 0.9 (Fig. 1). As with metoprolol, this equal permeation in both directions without the pH-gradient demonstrates that the permeation was mainly passive and the *P*-glycoprotein was not active in this batch of the cells. The polarised permeation under the pH-gradient conditions is the likely result of a higher fraction of the less permeable ionised form at the acidic apical pH in the AP–BL direction [27] and increased sink-conditions (reduced back flux) during BL–AP permeation [36]. The AP–BL transport of ketoprofen and ranitidine, which are mainly passively permeating compounds under iso-pH 7.4 conditions, remained at similar levels between the different Caco-2 batches. The Papp values in the P42 batch were $32.6 \pm 3.9 \times 10^{-6}$ cm/s (vs. $32.3 \pm 1.1 \times 10^{-6}$ cm/s) for ketoprofen and $1.63 \pm 0.12 \times 10^{-6}$ cm/s (vs. $1.31 \pm 0.20 \times 10^{-6}$ cm/s) for ranitidine.

These results show that verapamil could be used as a marker molecule for *P*-glycoprotein, as suggested by the

FDA, and point out the importance of having such a tool. However, more specific and sensitive marker molecules for P-gp, e.g., radiolabelled digoxin [37] or vinblastine [38], might better lend themselves for this purpose. Information about *P*-glycoprotein functionality in a cell line is essential when assessing the permeability of drug candidates/putative efflux-pump substrates.

3.3. Low permeability compounds

Of the low permeability compounds, the permeation of ranitidine was dependent on the pH according to its basic nature (Table 2, Fig. 1). Ranitidine has been reported to increase the TEER-values by blocking the paracellular anionic sites and to decrease even its own permeability [39,40]. Transport of ranitidine by the paracellular route occurs by means of both saturable and non-saturable mechanisms [40,41]. In the saturable component, facilitated diffusion of ranitidine occurs via interaction with the anionic sites of the paracellular space and is then translocated across the cell monolayers. The molecules which do not interact with the anionic sites are transported by passive paracellular diffusion. In the present study, these interactions in the paracellular space might explain the lower permeation of hydrochlorothiazide in cocktails compared to single permeability. In addition to paracellular interactions, interactions of ranitidine with *P*-glycoprotein substrates/inhibitors, like verapamil in this study, are also possible [21,22] This potential involvement of *P*-glycoprotein mediated efflux in ranitidine transport was evidenced by a lower BL–AP permeability in the cocktail ($0.95 \pm 0.16 \times 10^{-6}$ cm/s) compared to single compound permeability ($2.99 \pm 0.23 \times 10^{-6}$ cm/s) Hence, the efflux ratios of ranitidine in single and cocktail experiments dropped from 2.3 to 0.81, respectively, giving now different information of the transport mechanism.

Overall, the possible interactions with *P*-glycoprotein substrates and paracellular diffusion preclude the use of ranitidine as an internal permeability marker and raise the question about its suitability in standardising cocktails. Hydrochlorothiazide might be a good pH-independent marker for low permeability drugs as an internal standard, but as mentioned previously, its lower permeability in cocktails (putative interaction with ranitidine) needs further investigations (Table 2).

Fluorescein was probed as an alternative for mannitol. Fluorescein has been used as a marker molecule in the studies of paracellular absorption and leakage under iso-pH 7.4 [42,43]. The results of Konishi et al. indicated that the permeation of fluorescein at pH 7.4 was inversely related to TEER-values. This supports the assumption of paracellular absorption of fluorescein under iso-pH 7.4. The negative $\log D_{7.4}$ value of −0.36 (Table 1) further predicts the poor permeation. However, under pH-gradient conditions where acidic apical pH is utilised and the monoanionic form prevails, active transport has been reported (similar to ketoprofen) [24,25]. The average Papp value of fluorescein in all the AP–BL studies (both single and cocktail experiments) under iso-pH 7.4 was $0.37 \pm 0.07 \times 10^{-6}$ cm/s ($n = 10$) and the corresponding average for mannitol (incubation after the experiment) was $0.43 \pm 0.07 \times 10^{-6}$ cm. In the case of damaged monolayers, both fluorescein and mannitol gave clearly higher but similar Papp values for these monolayers (results not shown). The permeation of fluorescein in the BL–AP direction was higher than in the AP–BL direction. These results confirm the utilisation potential of fluorescein as an alternative for mannitol in the AP–BL direction without the pH-gradient. It can also be used in the n-in-one cocktails and it is easily analysed by fluorescence plate reader (provided that the other compounds involved do not contain similar fluorophore properties). Under pH-gradient conditions, however, the permeation of fluorescein was high in the AP–BL direction ($>25 \times 10^{-6}$ cm/s) compared to the BL–AP direction ($>0.2 \times 10^{-6}$ cm/s) (Fig. 1). The decrease in permeability in the presence of ketoprofen further points out the putative influence of active transport mechanisms.

3.4. The effect of insert size on drug permeability

Comparisons between insert sizes were performed for both single compound and cocktail-dosing studies under both pH conditions and in both directions with no systematic differences in permeation between the 12- and 24-well formats. The r^2-values of the insert size correlations both for AP–BL and BL–AP permeabilities were between 0.864 and 0.999, and the corresponding slopes of the plots were close to one (Table 3). In the case of pH-gradient conditions, the AP–BL transport of ketoprofen was variable, which attenuated the correlation values in single and in cocktail studies. The low correlation (high slope-values and low r^2-values in Table 3) were putatively a result of variable expression of MCT transporters between the passages in the Caco-2 cells (assuming active ketoprofen transport by an H^+ dependent transporter). In addition, high variability ($\geqslant 15\%$) in the between-day and within-day precision of ketoprofen in sample analysis [9] may have contributed to this. Without ketoprofen the slope and r^2-value were 1.11 and 0.904 in the single compound study and 1.37 and 0.999 in the cocktail-dosing study, respectively.

Table 3
The correlation coefficients (r^2) and slopes of the linear regression between the Papp values of the model compounds across 0.33 cm^2 and 1.1 cm^2 monolayer inserts

	Apical pH 7.4		Apical pH 5.5	
	r^2	Slope	r^2	Slope
AP–BL single	0.999	1.020	0.864	2.004
AP–BL cocktail	0.969	1.165	0.914	1.564
BL–AP single	0.923	0.755	0.953	0.916
BL–AP cocktail	0.992	0.983	0.954[a]	0.979[a]

[a] Without fluorescein.

4. Conclusions

These results confirm that cocktail-dosing is a completely feasible validation method to ensure that the Caco-2 cell line behaves in a determined and predictable way in the permeability tests. Even though sink conditions were not present for highly permeable compounds in cocktail experiments, the rank order correlations and the classification to low and high permeability compounds were successful when compared to the values of single compound experiments. In addition, this study proved that the permeability values of 12- and 24-well formats are directly comparable to each other. The changing permeability of verapamil between the cell batches clearly demonstrated the necessity of standardisation for each batch when studying potential *P*-glycoprotein candidates. Depending on the compounds used and the pH-conditions, more specific mechanistic evaluations (transcellular and paracellular passive permeation, active transport, P-gp efflux) can be included in the studies. Of the compounds studied, especially antipyrine, metoprolol and hydrochlorothiazide (+mannitol) have repeatable permeability characteristics and can be highly recommended for evaluation and standardisation purposes of Caco-2 cell monolayers.

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