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Effect of simulated intestinal fluid on drug permeability estimation across Caco-2 monolayers

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Abstract

Presently, the Caco-2 cell culture model is widely used during drug discovery and development as a predictive tool for the oral absorption of drug candidates. For transport experiments in the Caco-2 system, HBSS-like buffered salt solutions are commonly used, although different shortcomings have been associated with the use of these buffers. In this paper, we investigated the effect of using fasted state simulated intestinal fluid (FaSSIF) as potential biorelevant medium for the drug permeability estimation across Caco-2 monolayers. The transport characteristics of 19 model compounds were determined in the Caco-2 cell culture model in the presence of FaSSIF as compared to classic transport medium. A sigmoidal relation was obtained when the estimated P_{app}, s of the apical to basolateral transport were plotted versus the reported values of the fraction absorbed in man. Although no effect of FaSSIF as compared to classic transport medium (TM) was observed on the total predictability of the model, an impact was demonstrated (1) on the bi-directional transport of actively transported drugs (including talinolol, digoxin and doxorubicin), (2) on recovery and (3) on the solubility and permeability estimation of poorly water-soluble drugs. The observed differences may be attributed to a P-gp inhibitory effect of sodium taurocholate (NaTC), micellar encapsulation by the NaTC/lecithin mixed micelles and/or an increase of the solubility of lipophilic drugs. As the experimental conditions should mimic the physiological in vivo conditions, the use of FaSSIF as medium during Caco-2 experiments may improve the biorelevance of the model.

Keywords: Caco-2; Fasted state simulated intestinal fluid (FaSSIF); Permeation; Fraction absorbed

1. Introduction

The transepithelial transport of compounds is an important characteristic, commonly assessed during the evaluation and selection of potential drug candidates. The Caco-2 cell culture model was introduced in the early 1990s and has become a widely used tool for the determination of the intestinal transport characteristics of compounds (Hidalgo et al., 1989; Hilgers et al., 1990; LeCluyse and Sutton, 1997; Gan and Thakker, 1997). Several reports have demonstrated the possibility to predict the oral absorption of drugs

Abbreviations: A-to-B, apical to basolateral; BCRP, breast cancer resistance protein; B-to-A, basolateral to apical; CsA, cyclosporin A; D, donor compartment; Fa, human fraction absorbed; FaSSIF, fasted state simulated intestinal fluid; HBSS, Hanks' balanced salt solution; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulphonic acid; MRP, multidrug resistance-associated protein; NaTC, sodium taurocholate; P_{app}, apparent permeability coefficient; R, receiver compartment; TEER, transepithelial electrical resistance; TM, transport medium

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in man based on their permeability observed in Caco-2 monolayers (Artursson and Karlsson, 1991; Artursson et al., 2001). Within the framework of the Biopharmaceutic Classification System (BCS), the rate of mass transfer of a compound across the Caco-2 monolayer can even be considered to allow a waiver for in vivo bioequivalence studies (Yu et al., 2002). However, many discrepancies in culturing and experimental conditions can be identified in the literature related to Caco-2 maintenance and experiments.

For the realization of permeability and transport studies on the Caco-2 cell culture model, classic buffered salt solutions are commonly used (e.g. Hanks' balanced salt solution (HBSS) buffered with HEPES (10 mM) at pH 7.4 and supplemented with glucose). Nevertheless, many shortcomings are associated with the use of such saline buffers for Caco-2 experiments, including the limited solubility of highly lipophilic drugs, the adsorption and/or non-specific binding to the device surfaces or (in)to the cells and the poor physiological relevance of the media used. To overcome these issues, several categories of media (for the apical and basolateral compartment), including plain salt solutions composed of inorganic salts and glucose, culture medium and solvents mimicking intestinal fluid have been proposed and have recently been reviewed (Ingels and Augustijns, 2003).

We investigated the possibility of using fasted state simulated intestinal fluid (FaSSIF) as apical solvent for Caco-2 experiments. FaSSIF has originally been introduced by the group of Professor Dressman in 1998 as dissolution medium to simulate the in vivo dissolution behavior of compounds (Galia et al., 1998; Dressman et al., 1998; Dressman and Reppas, 2000). The composition of FaSSIF is shown in Table 1. It has previously been shown that FaSSIF buffer was compatible with the Caco-2 cell monolayer for at least 2 h. The transport of different model compounds (i.e. theophylline (passive diffusion), phenylalanine (active transport)) and the activity of the brush border enzyme aminopeptidase were similar when using classic TM and FaSSIF. However, a concentration-dependent P-gp inhibitory activity of sodium taurocholate (NaTC) (present in FaSSIF) when assessing cyclosporin A (CsA) transport was demonstrated (Ingels et al., 2002a).

Table 1
Composition of fasted state simulated intestinal fluid (FaSSIF) as published by Dressman and Reppas (2000)

FaSSIF	
Sodium taurocholate (NaTC)	3 mM
Lecithin	0.75 mM
$NaH_2PO_4 \cdot H_2O$	3.9 g
NaCl	6.2 g
NaOH	ad pH 6.5
Water	ad 1 l

The aim of the present study was to evaluate the impact of FaSSIF as potential biorelevant medium on the permeability estimation and transport characteristics of drug compounds in the Caco-2 cell culture model.

2. Materials and methods

2.1. Materials

Sodium taurocholate (NaTC) was purchased from Fluka (Bornem, Belgium). Phospholipon 90G was provided by Nattermann Phospholipid GmbH (Köln, Germany). Atenolol, digoxin, sulfasalazine, phenylalanine, progesterone, lidocaine, indomethacin, prazosin, theophylline, chlorothiazide, danazol, furosemide, verapamil, doxorubicin and propranolol were from Sigma (Bornem, Belgium). Talinolol was kindly provided by AWD Pharma GmbH & Co., Dresden, Germany. All solvents used for analysis were HPLC grade. Purified water was used for all aqueous solutions and mobile phases. All chemicals used for the Caco-2 culture and cell culture supplies, including Nunc's tissue culture inserts with Anopore™ (Whatman Scientific Ltd.) membrane (0.2 μm pore size, 25 mm diameter) were purchased from Invitrogen (VWR International, Leuven, Belgium). Transwell inserts (0.4 μm pore size, 12 mm diameter) were obtained from Corning-Costar (VWR International, Leuven, Belgium). Culture medium consisted of Dulbecco's modified Eagle medium (DMEM) containing 100 IU/ml penicillin–100 μg/ml streptomycin, 1% MEM non-essential amino acids and 10% fetal bovine serum. Transport medium (TM) consisted of HBSS supplemented with glucose (final concentration 25 mM) and HEPES (10 mM) adjusted with NaOH 0.2N to pH 7.4 or 6.5. All other chemicals were of the highest purity and were used as received.

Table 2
Reference set of drug compounds with fraction absorbed data and BCS classification

Compounds	Fraction absorbed (%)	BCS class
Atenolol	50 (37–71)[a,b,c,d,e,f,g]	III
Chlorothiazide	13 (10–56)[a,c,h]	IV[h,i]
Danazol		II[c,j]
Digoxin		
Doxorubicin	5[a]	
Fluoxetine	80[k]	
Furosemide	61 (50–61)[a,c,f,h]	III[h,i]
Indomethacine	100[a]	
Ketoconazole		II
Lidocaine		
LY334370	100	
Phenylalanine	100[l]	I
Prazosin	100[a]	
Progesterone	91[a,c]	
Propranolol HCl	90 (90–99)[a,c,e,g,h]	I[h]
Sulfasalazine	13 (7–17)[a,b,e,g]	
Talinolol		
Theophylline	98 (96–100)[a,f,g]	I
Verapamil	100[a]	I[c]

[a] Zhao et al. (2001).
[b] Stenberg et al. (2001).
[c] Irvine et al. (1999).
[d] Lennernäs et al. (1996).
[e] Artursson and Karlsson (1991).
[f] Yamashita et al. (2000).
[g] Liu et al. (2002).
[h] Pade and Stavchansky (1998).
[i] Taub et al. (2002).
[j] Galia et al. (1998).
[k] Lemberger et al. (1985).
[l] Löbenberg and Amidon (2000).

2.2. Reference compounds

Nineteen drug compounds were selected (Table 2) based on our intention to cover the whole range of fraction absorbed values in man, on their availability, on the availability of Fa data in literature and for their diversity in expected transport mechanism. Five of the selected drugs (i.e. propranolol, theophylline, verapamil, atenolol and furosemide) were part of the list suggested by the FDA (CDER, 2000). To our knowledge, no human fraction absorbed data have been reported for talinolol, ketoconazole, danazol, lidocaine and digoxin. For talinolol and digoxin, the mean oral bioavailibility was reported to be 55% (Trausch et al., 1995) and 67% (Zhao et al., 2001), respectively. Danazol was demonstrated to have a low bioavailibility due to dissolution-rate-limited absorption and hepatic metabolism (Charman et al., 1993; Farag Badawy et al., 1996). Lidocaine is readily absorbed from the gastro-intestinal tract, while the oral absorption of ketoconazole is variable and increases with decreasing stomach pH (e.g. in combination with Coca-Cola) (Chin et al., 1995; Martindale, 1996). These compounds were included in the data set to evaluate the impact of FaSSIF as compared to classic TM on their transport characteristics.

2.3. Preparation of cell monolayers

Caco-2 cells were purchased from ATCC (Rockville, MD, USA) and grown in 75 cm^2 Nunc flasks in an incubator at 37 °C with controlled atmosphere containing 5% CO_2 and 90% relative humidity. Caco-2 cells were seeded on polycarbonate Transwell or Anopore™ filters at a density of 90,000 cells/cm^2. The maintenance and seeding of the Caco-2 cells were performed following previously published procedures (Augustijns et al., 1998; Ingels et al., 2002b). Passage numbers from 110 to 145 were used for experiments. Only confluent monolayers (22–24 days post-seeding) having TEER values above 200 Ω cm^2 were used in these experiments. All volumes added to the apical or basolateral compartment during experiments amounted to 0.5 and 1.0 ml (for 12-well plates) or 2.0 and 2.0 ml (for 6-well plates), respectively. All experiments were performed in Transwell inserts (0.4 μm pore size, 12 mm diameter), except for the cumulative transport assay with danazol, for which Anopore™ filter inserts were used (0.2 μm pore size, 25 mm diameter).

2.4. Transport experiments across Caco-2 monolayers

2.4.1. Standard procedure

Prior to each experiment, Caco-2 monolayers were first rinsed twice with TM. Inserts were then pre-incubated with TM for 60 min, after which TEER values were measured. The medium was replaced by TM or FaSSIF containing the test compound at the donor side. In all experiments, TM pH 7.4 was used as receiver medium, unless stated differently. All donor solutions contained 1% DMSO, except for the experiment with danazol, for which a suspension

of the compound in TM or FaSSIF was used. Donor concentration for the different compounds was standardized at 10 μM, except for sulfasalazine (100 μM), phenylalanine (1 mM) and atenolol (100 μM) because of analytical considerations. After an incubation period of 60 min (for propranolol, theophylline and phenylalanine) or 120 min (for the other compounds), samples were collected and TEER values were measured. Samples were analyzed by LC/UV or LC/MS. The HPLC system consisted of a Waters 2695 Separations module, combined with a Waters 2487 dual absorbance or Waters Micromass ZQ detector (Waters, Brussels, Belgium). After each experiment, an incubation step with sodium fluorescein (0.1%, w/v) was performed for 60 min, followed by TEER measurement, as an additional control of the integrity of the Caco-2 monolayer. The amount of sodium fluorescein appearing in the receiver compartment was measured by UV spectrophotometry at 490 nm. Sodium fluorescein flux values across the monolayers were below 0.5%/h cm^2. TEER values remained above 200 Ω cm^2.

2.4.2. Transport experiment in presence of inhibitors

In order to study the effect of competition on the P-glycoprotein efflux transporter (P-gp), the preincubation and the incubation step were performed in the presence of the inhibitor (LY335984 1 μM) included in both, the apical and basolateral compartment. In this experiment, FaSSIF was used as apical medium.

2.4.3. Cumulative transport of danazol

About 10 mg of danazol was suspended in 10 ml TM pH 6.5 or in 10 ml FaSSIF. Caco-2 monolayers (seeded on 25 mm Anopore™ membranes) were rinsed twice with TM and preincubated with TM for 60 min. To initiate the A-to-B transport, the medium was replaced by the danazol suspension in TM pH 6.5 or in FaSSIF at the apical side. The basolateral compartment consisted of TM pH 7.4. The inserts were transferred every 30 min to wells containing fresh TM. Plates were agitated on a orbital shaker at 50 rpm during the total incubation period of 120 min. Samples were analyzed by HPLC with UV detection (285 nm).

2.5. Solubility determination of danazol

About 2 mg of danazol were weighed in a clear glass vial. One milliliter of TM or FaSSIF were added to the vials, which were then agitated with a magnetic stirrer for 2 h at 37 °C. Suspensions were filtered on a Gelman 0.2 μm filter for aqueous solutions. The filtrate was analyzed by HPLC/UV (285 nm). The experiment was performed in triplicate.

2.6. Data analysis

2.6.1. Apparent permeability coefficient (P_{app})

The apparent permeability coefficient (in cm/s) was calculated as follows:

$$P_{\text{app}} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times C_0} \quad (1)$$

with $\Delta Q/\Delta t$ the amount of drug appearing in the receiver compartment in function of time (nmoles/s), C_0 the initial concentration in the donor compartment (μM) and A the surface area (cm^2) across which transport occurred.

2.6.2. Statistical analysis

Determined P_{app} values for the A-to-B transport (in TM and FaSSIF) were plotted against the human fraction absorbed (Fa) (obtained from literature). A model based on a sigmoid curve with two parameters, given by Eq. (2) (Ratkowsky, 1990; Artursson et al., 2001), was fitted to the P_{app} observations for both experimental set-ups:

$$\text{Fa} = 100 - \frac{100}{1 + \exp[G \log(P_{\text{app}} AB/E)]} \quad (2)$$

with E the P_{app} level associated to 50% of fraction absorbed and G the slope of the curve at the inflexion point of the curve.

Best fitting curves were calculated by using the nonlinear least square method (build-in function nls of S-plus 6.1 software, Insightful, WA, USA).

2.6.3. Recovery

The recovery is defined as the amount recovered in the apical and basolateral compartment at the end of the experiment and is expressed as a percentage of the amount added to the donor side at time zero (Eq. (3)):

$$\text{Recovery} = \frac{(C_{\text{R,120 min}} \times V_{\text{R}} + C_{\text{D,120 min}} \times V_{\text{D}})}{C_{\text{D,0 min}} \times V_{\text{D}}} \times 100 \quad (3)$$

with $C_{\text{R,120 min}}$ and $C_{\text{D,120 min}}$ the concentration measured after 120 min in the receiver and donor compart-

ment, respectively, $C_{D,0\,min}$ the concentration of the test compound in the donor compartment at time zero and V_R and V_D the volumes buffer added in receiver and donor compartment, respectively.

3. Results and discussion

3.1. Evaluation of transport characteristics of model compounds

3.1.1. Bi-directional assays

Model compounds (Table 2) were evaluated for their bi-directional transport in the Caco-2 experimental set-up with FaSSIF as donor medium as compared to classic TM. The A-to-B and B-to-A P_{app} was calculated following Eq. (1) for all the compounds dissolved in either FaSSIF or TM (donor medium). The apparent permeability coefficients (cm/s) obtained for the A-to-B and B-to-A transport of the test compounds are summarized in Table 3.

The presence of FaSSIF as donor solvent did not influence the drug permeation of atenolol, indomethacin, phenylalanine, progesterone and theophylline. A decrease of the A-to-B transport with FaSSIF as donor solvent as compared to TM was observed for danazol (20.2-fold), fluoxetine (17.1-fold), ketoconazole (1.5-fold), lidocaine (3.6-fold), LY334370 (2.7-fold), prazosin (2.1-fold), propranolol (3.0-fold) and verapamil (9.9-fold). A decrease in B-to-A transport was also observed when FaSSIF was used as donor solvent in the basolateral compartment: danazol (11.1-fold), fluoxetine (6.9-fold), ketoconazole (1.4-fold), lidocaine (2.2-fold), prazosin (1.7-fold), propranolol (3.5-fold) and verapamil (4.7-fold). A decrease of only the B-to-A transport was observed for the P-gp substrates (digoxin (2.1-fold), doxorubicin (3.5-fold) and talinolol (25.8-fold)) and for the drugs chlorothiazide (2.0-fold), furosemide (3.2-fold) and sulfasalazine (1.6-fold). For these compounds, the decrease in B-to-A transport in presence of FaSSIF at the donor side resulted in a lower B-to-A/A-to-B ratio when using FaSSIF as compared to TM. The reduction of this B-to-A/A-to-B ratio is shown for the P-gp substrates in Fig. 1.

Table 3
Determined P_{app} values for the A-to-B and B-to-A transport of the drug compounds in presence or absence of FaSSIF (as donor solvent)

Compounds	TM pH 6.5		FaSSIF pH 6.5	
	P_{app} A-to-B ($\times 10^{-6}$ cm/s)	P_{app} B-to-A ($\times 10^{-6}$ cm/s)	P_{app} A-to-B ($\times 10^{-6}$ cm/s)	P_{app} B-to-A ($\times 10^{-6}$ cm/s)
Atenolol (100 μM)	0.09 (±0.01)	0.09 (±0.05)	0.12 (±0.04)	0.16 (±0.03)
Chlorothiazide	*0.05 (±0.01)*	*1.33 (±0.12)*	*0.07 (±0.02)*	*0.67 (±0.06)*
Danazol	14.15 (±1.23)	7.81 (±1.11)	0.73 (±0.23)	0.67 (±0.05)
Digoxin	**0.45 (±0.02)**	**14.80 (±2.06)**	**0.46 (±0.03)**	**6.91 (±0.45)**
Doxorubicin	**0.28 (±0.03)**	**2.07 (±0.15)**	**0.29 (±0.01)**	**0.59 (±0.12)**
Fluoxetine	15.40 (±2.92)	13.10 (±1.26)	0.86 (±0.51)	1.86 (±0.50)
Furosemide	*0.12 (±0.04)*	*6.78 (±0.40)*	*0.27 (±0.04)*	*2.08 (±0.05)*
Indomethacin	38.30 (±0.80)	22.30 (±0.36)	39.40 (±2.41)	17.80 (±0.64)
Ketoconazole	15.10 (±1.23)	11.00 (±0.95)	9.65 (±0.35)	7.55 (±0.69)
Lidocaine	18.50 (±0.20)	13.10 (±0.86)	5.12 (±0.41)	5.99 (±0.16)
LY334370	4.76 (±0.91)	20.70 (±0.81)	1.75 (±0.11)	22.50 (±0.71)
Phenylalanine (1 mM)	6.8 (±0.4)	0.7 (±0.2)	6.1 (±0.4)	0.5 (±0.1)
Prazosin	10.30 (±0.55)	17.60 (±1.44)	4.99 (±0.28)	10.50 (±0.43)
Progesterone	20.80 (±2.09)	13.60 (±2.79)	17.00 (±0.27)	13.10 (±0.92)
Propranolol HCl	13.70 (±0.41)	11.60 (±1.00)	4.53 (±0.63)	3.31 (±0.19)
Sulfasalazine (100 μM)	*0.08 (±0.02)*	*12.00 (±0.53)*	*0.06 (±0.01)*	*7.43 (±0.23)*
Talinolol	**0.28 (± 0.08)**	**6.38 (±0.50)**	**0.34 (±0.08)**	**0.30 (±0.02)**
Theophylline	23.2 (± 0.3)	26.6 (±0.6)	28.0 (±0.4)	27.2 (±0.4)
Verapamil	29.60 (±2.44)	16.60 (±3.91)	2.95 (±0.65)	3.49 (±0.24)

P-gp substrates are given in bold. Effluxed compounds (by non-P-gp or non-defined efflux carriers) are written in italic. TM pH 7.4 was used as receiver medium. All compounds were tested at 10 μM unless stated differently. The values represent the average value of three experiments (±S.D.).

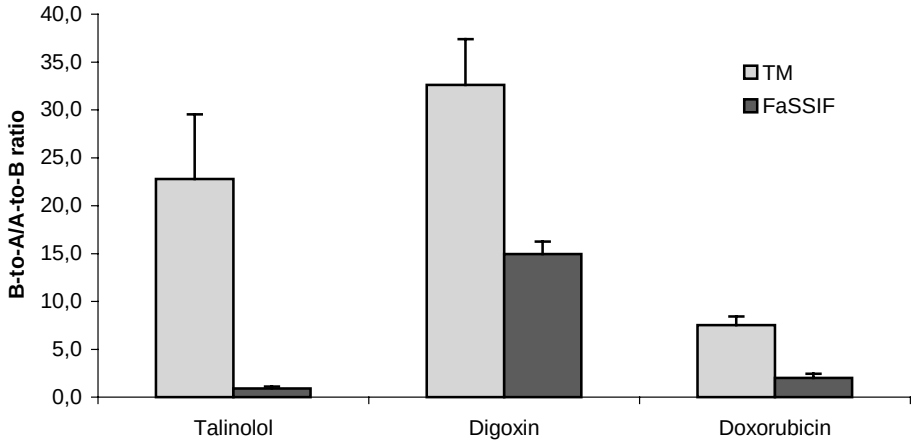

Fig. 1. B-to-A/A-to-B ratio for the P-gp substrates talinolol, digoxin and doxorubicin. Bars represent the mean value of $n = 3$. FaSSIF was used as donor medium.

Overall, the effect of FaSSIF on the bi-directional transport of drug compounds can be summarized as presented in Table 4. For passively transported drug compounds, both the A-to-B and the B-to-A transport are similar or reduced in presence of FaSSIF as compared to TM. For the P-gp substrates and other effluxed compounds, the B-to-A transport is reduced when tested in FaSSIF as compared to TM, resulting in a decrease of the B-to-A/A-to-B ratio. The A-to-B transport of these compounds was similar in both media or higher in FaSSIF (e.g. furosemide).

3.1.2. Micellar encapsulation and/or modulation of carriers

The decrease in transport of drug compounds in presence of FaSSIF may be explained by encapsulation or interaction of the drug compounds into or with the NaTC/lecithin mixed micelles (present in FaSSIF), as only the free concentration of the drug is available for transport through the cell monolayer. In addition, NaTC may interfere with (active) carriers located at the cell membrane. NaTC has for instance been reported to have a P-gp inhibitory effect. When FaSSIF or dilutions of FaSSIF with TM were used in the apical compartment, we demonstrated a concentration-dependent increase in absorptive transport, as well as a concentration-dependent decrease of the secretory transport of CsA (Ingels et al., 2002a). In contrast to these previous observations, no increase in absorptive transport of the P-gp substrates talinolol, doxorubicin and digoxin in presence of FaSSIF as compared to TM could be observed.

The difference of effect for these compounds as compared to the effect observed for CsA may be explained by a difference in affinity of the different compounds for the P-gp carrier and/or the NaTC/lecithin mixed micelles. The potential effect of NaTC on other efflux transporters has not been reported yet. The significant decrease of the B-to-A transport of doxorubicin, chlorothiazide, sulfasalazine and furosemide may possibly be caused by an interference of components present in FaSSIF on other efflux carriers in addition to micellar encapsulation. Sulfasalazine has been demonstrated to be effluxed by a variety of efflux transporters including at least MRP and an anion sensitive transport system (Liang et al., 2000), while furosemide was shown to be effluxed by an indomethacin-dependent efflux transporter (Flanagan and Benet, 1999). Doxorubicin has been reported as a P-gp and BCRP (breast cancer resistance protein)

Table 4
Observed/expected effect of FaSSIF on the determined P_{app} values and B-to-A/A-to-B ratio of passively transported and actively effluxed (P-gp and non-P-gp) drug compounds as compared to TM

Transport category	P_{app} A-to-B	P_{app} B-to-A	B-to-A/ A-to-B ratio
Passive	=/↓	=/↓	=
Efflux (P-gp and non-P-gp)	=/↑	↓	↓

substrate (Zhang et al., 1995; Lage and Dietel, 2000). It is interesting to note that all drugs which have been described to be a substrate for efflux transporters did not show a decrease in absorptive transport as observed for most of the passive diffusion compounds. One might speculate that the absence of a decrease in A-to-B transport, which was explained by micellar encapsulation, is compensated for by a simultaneously ongoing inhibition of the modulatory effect of efflux carriers by components present in FaSSIF (e.g. NaTC).

In order to discriminate between the potential micellar encapsulation and P-gp modulation effect of FaSSIF when assessing the transport of P-gp substrates, we investigated the transport of talinolol in presence or absence of FaSSIF (at the apical side) and in presence or absence of the selective P-gp inhibitor LY335984. The results are shown in Fig. 2. In presence of the inhibitor LY335984, we observed a loss of the polarity in transport for the control condition (B-to-A/A-to-B ratio of 1.2), while in FaSSIF as apical medium a polarity factor of 2.3 is still present. The asymmetry in transport observed in presence of the P-gp inhibitor LY335984 when performing the experiment in FaSSIF indicates that an additional parameter (i.e. micellar encapsulation) may influence the transport of talinolol. On the other hand, the inhibitor itself could be (partly) encapsulated and consequently have less inhibitory activity.

3.2. Relation with human fraction absorbed (Fa)

The observed absorptive apparent permeability coefficients were plotted versus the reported values of the fraction absorbed in man (Fig. 3) for both TM and FaSSIF as donor medium. The best fitting curves for data generated with TM or FaSSIF as apical buffer were calculated and optimized following Eq. (2). The parameters for the best fitting curves were $E = 2.82 \times 10^{-7} \pm 1.27 \times 10^{-7}$ cm/s and $G = 0.73 \pm 0.23$ for the experimental set-up with TM and $E = 2.98 \times 10^{-7} \pm 7.25 \times 10^{-8}$ cm/s and $G = 1.16 \pm 0.32$ for the FaSSIF model. For both media, the obtained relation was sigmoidal, similar as the relationship previously reported by Artursson and Karlsson (1991). As the respective confidence bands were overlapping, there was no evidence for a significant difference between the two fitted curves. Based on our assay results (Fig. 3), a $P_{app} \leq 4.18 \times 10^{-8}$ cm/s and $\leq 9.00 \times 10^{-8}$ cm/s were considered as predictive for poor oral absorption (0–20%) and a P_{app} value $\geq 9.07 \times 10^{-7}$ and $\geq 6.21 \times 10^{-7}$ cm/s were predictive for good oral absorption (70–100%) for the experimental set-up with TM and FaSSIF, respectively. Notwithstanding the observed decrease in A-to-B transport when using FaSSIF as compared to TM for a number of reference compounds, no difference was observed when classifying the majority of these compounds with respect to their

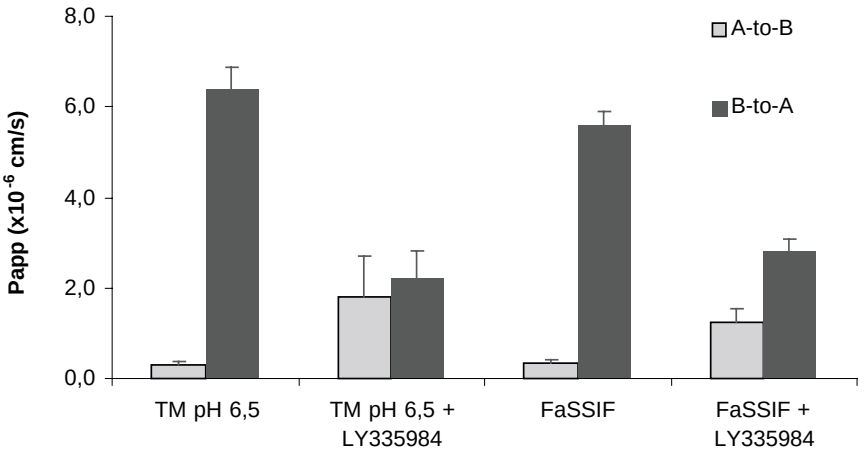

Fig. 2. Transport of P-gp substrate talinolol (10 μM) in TM pH 6.5, FaSSIF and in presence or absence of the LY335984 (1 μM) in TM pH 6.5 and FaSSIF, respectively. The basolateral medium consisted of TM buffered at pH 7.4. In presence of LY335984 (1 μM), the inhibitor was included in both, the apical and basolateral, compartment. Bars represent mean values ($n = 3$) + S.D.

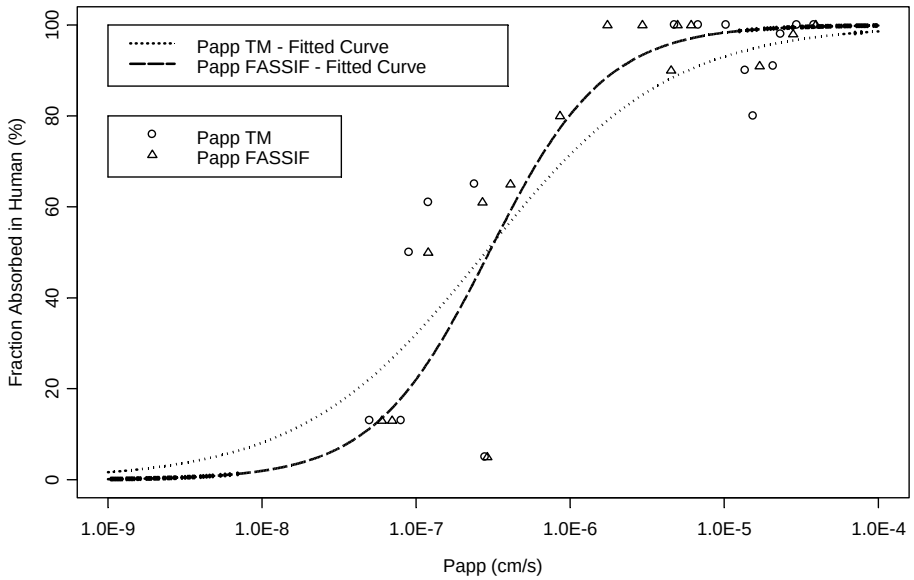

Fig. 3. Relation between the fraction absorbed of orally administered drugs in man and their apparent permeability coefficients in the Caco-2 model in presence (Δ) or absence (○) of FaSSIF buffer. Data points represent mean values ($n = 3$). The pH value of the apical medium was in both cases adjusted to pH 6.5. The basolateral solvent consisted of TM buffered at pH 7.4. Lines represent best fitting curves for the model used with TM (. . .) or FaSSIF (– – –).

absorption potential based on apparent permeability coefficient values when using either FaSSIF or TM. In Fig. 4, the predicted human Fa of each drug was recalculated from its P_{app} value in the Caco-2 study and was plotted against the observed Fa in human. For most of the tested compounds, no difference was observed when the Caco-2 study was carried out with FaSSIF as apical solvent as compared to the control condition using TM. However, a difference in classification was observed for two compounds (i.e. chlorothiazide and sulfasalazine). Based on the determined sigmoidal curves, chlorothiazide and sulfasalazine are correctly predicted to have a poor oral absorption considering the P_{app} determined with FaSSIF as apical medium, while they are expected to be moderately absorbed if TM is used. In both models, the calculated Fa of doxorubicin was overestimated. This discrepancy might be explained by an underestimation of the BCRP efflux component when assessing the in vitro transport of doxorubicin.

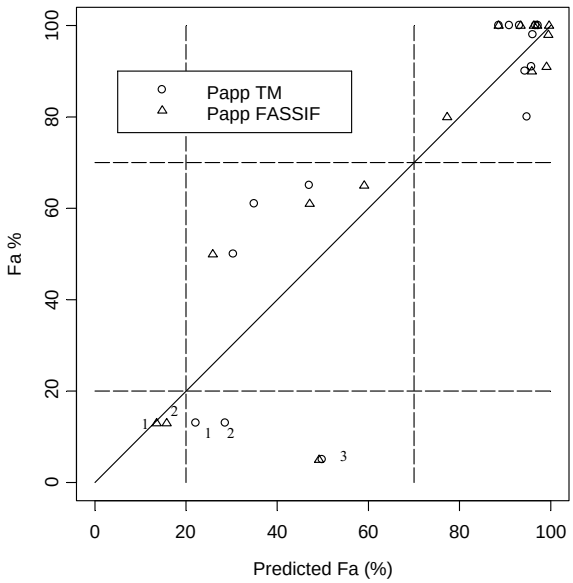

Fig. 4. Predicted Fa vs. the reported Fa for the model used with TM (○) or FaSSIF (Δ) with respect to the ideal diagonal case. (1) Chlorothiazide, (2) sulfasalazine and (3) doxorubicin. The predicted value of the absorbed fraction was obtained from the P_{app} of each drug based on the relationship defined in Eq. (2).

3.3. Effect on the permeation of danazol

Although the use of FaSSIF did not impact the global predictive value of the model, it was mentioned

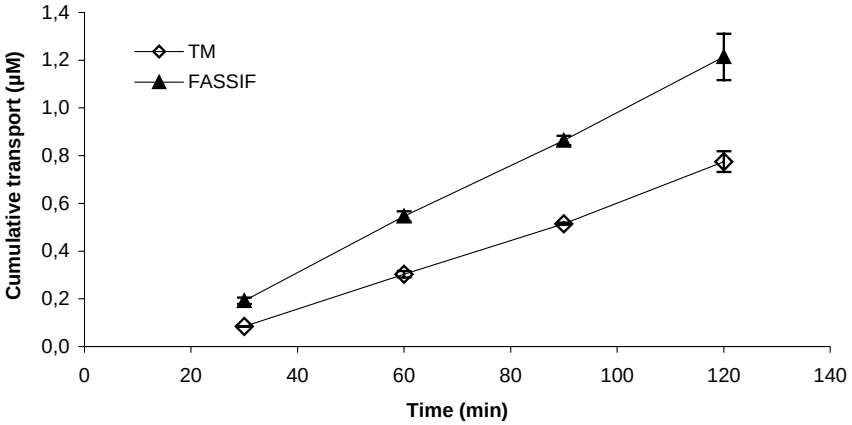

Fig. 5. Cumulative A-to-B transport of danazol from a suspension of danazol in TM or in FaSSIF (in absence of DMSO). TM pH 7.4 was used as receiver medium. Data points represent mean values of 3 ± S.D.

before that the use of FaSSIF as donor solvent during Caco-2 experiments could impact active transport mechanisms of different drugs. In addition, NaTC has been reported to have good solubilization properties and to affect the permeability of compounds (Taub et al., 2002; Udata et al., 2003). In order to investigate the potential benefits of FaSSIF for the solubilization of the test compounds, we studied the A-to-B transport of a suspension of danazol in TM or FaSSIF in absence of the co-solvent DMSO. As shown in Fig. 5, the cumulative transported amount of danazol was increased when FaSSIF was used as compared to TM. This increase was caused by the higher solubilized fraction of danazol in presence of FaSSIF. The solubility of danazol in TM and FaSSIF was equal to 0.05±0.01 μg/ml and 6.20±0.04 μg/ml, respectively.

3.4. Recovery

Recovery is defined as the amount recovered in the apical and basolateral compartment at the end of the experiment, expressed as a percentage of the amount added at time zero (Eq. (3)). For the determination of the recovery, the intra-cellular concentration of a drug compound is not taken into account, as at very early stages of development, no validated analytical methods are available to analyze drug compounds in biological matrices. An acceptable recovery value at the end of the Caco-2 experiment is critical for the predictive value of the Caco-2 experiment. Indeed, if a low recovery is obtained (e.g. by entrapment of the compound into the cell monolayer or by adsorption to the device surface), this could result in an erroneous estimation of the transport rate of the compound. The recovery will become even more crucial when active transport mechanisms are involved. It has been reported that, for efflux substrates, conditions that create high cellular accumulation of drug could result in an overestimation of the secretory transport, so that the effects of this efflux as an impediment to drug absorption could be overestimated (Aungst et al., 2000). On the other hand, it could be argued that a higher cellular concentration could saturate the efflux mechanism involved and so lead to less (concentration-normalized) efflux. To evaluate the impact of the use of FaSSIF as compared to the use of TM on the recovery after transport experiments, we selected eight compounds of variable lipophilicity based on their (calculated) log *P* value (Table 5). As shown in Table 5, a significant correlation of the recovery (determined in TM) in function of the (calculated) log *P* value of the tested compounds was observed. We clearly observed a decrease of the recovery when using TM buffer for compounds with moderate to high log *P* values (above 2.5), while when performing the assay with FaSSIF as buffer, the recovery values remained acceptable. In this case, the calculated recoveries remained >71% for the whole range of tested compounds. The higher recovery obtained when FaSSIF is used as apical buffer as compared to TM could be explained by a modification of the partitioning behavior of the more lipophilic drugs between the micelles present in the

Table 5
Reported log *P* (calculated or measured) and recovery values (%) for the A-to-B transport experiment of the compounds in presence or absence of FaSSIF

Compound	(c) log *P*[a,b,c]	Recovery	
		TM (%)	FaSSIF (%)
Doxorubicin	−1.45	90.3	82.3
Chlorothiazide	−0.31	93.5	96.1
Lidocaine	2.26	81.6	88.8
Prazosin	2.45	48.0	87.2
Propranolol HCl	2.75	58.6	80.8
Progesterone	3.78	43.6	71.8
Indomethacine	4.18	56.6	104.9
Ketoconazole	4.45[b]	44.6	90.6

All compounds were tested at 10 μM. The values represent the average value of three experiments.
[a] Zhao et al. (2001).
[b] Braun et al. (2000).
[c] Lipinski et al. (2001).

apical buffer when FaSSIF is used and the cell monolayer. FaSSIF may also prevent the adsorption of compounds to the plastic device surfaces.

4. Conclusions

In this paper, we evaluated the effect of using FaSSIF buffer as medium for performing Caco-2 experiments. Although the use of FaSSIF as compared to TM did not affect the global predictive value of the model, an impact was shown on (1) the permeation determination of actively transported drugs (including talinolol, digoxin, doxorubicin, sulfasalazine and furosemide), (2) on the solubility and permeation of the poorly water-soluble drug danazol and (3) on the recovery values of more lipophilic drugs.

The observed differences may be attributed to micellar encapsulation by the NaTC/lecithin mixed micelles, an increase of the solubility of lipophilic drugs, and/or a P-gp inhibitory effect of NaTC. The real contribution of bile salts in the in vivo absorption process remains to be elucidated. The effect of NaTC or other components of intestinal fluid on the transport of drug compounds will be dependent on the affinity of the drug for the mixed micelles, the dose and the carriers involved. As these parameters are not necessarily known, especially in very early development stages, the relative contribution of the micellar encapsulation and the effect of NaTC on active carriers cannot be predicted. Nevertheless, we believe that the use of FaSSIF could improve the physiological relevance of the Caco-2 cell culture model. However, we do not recommend the use of FaSSIF for mechanistic studies. For this type of experiments, the apical and basolateral solvents should be similar or identical in order to exclude any external parameters of influence on the bi-directional transport of drugs.

On the other hand, we are convinced that, for the prediction of the oral absorption potential of compounds based on their A-to-B transport in the Caco-2 cell culture system, the experimental in vitro conditions should mimic the physiological conditions. The use of FaSSIF as apical solvent during Caco-2 experiments in drug discovery programs may improve the biorelevance and quality of the model because of the higher solubility, higher recovery and its possible effect on different carrier systems.

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