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Bio-relevant media to assess drug permeability: Sodium taurocholate and lecithin combination or crude bile?

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ABSTRACT

The assessment of *in vivo* drug absorption with *in vitro* permeability models demands the use of transport media with surface acting compounds. With the aim to establish their influence on *in vitro* permeability of 30 drugs through Caco-2 monolayers, cell vitality/integrity and micellar drug entrapment, taurocholate/lecithin (NaTC/Leci) and pig crude bile were applied. Drug permeabilities were correlated to fraction of drugs absorbed and appropriate NaTC/Leci and bile concentrations were proposed to simulate fasted/fed conditions *in vitro* (bile in the concentration range 1–5 v/v% or 0.2/0.05 mM NaTC/Leci for fasted; 10 v/v% bile or 3/0.75 mM NaTC/Leci for fed conditions) without detrimental effects on monolayer integrity/vitality (NaTC/Leci was more toxic than bile). Surfactants exerted different affinities for drugs; free drug concentration (c_{free}) of some was significantly lowered only by bile, while for the others NaTC/Leci and bile significantly diminished c_{free}. For some substances NaTC/Leci and bile significantly increased their permeabilities (*i.e.* more than 3-times) in spite of profound c_{free} decrease indicating the existence of an alternative absorption mechanism. Based on these data, the impact of bile on *in vitro* drug permeability and micellar drug entrapment cannot be adequately simulated by NaTC/Leci, because their effects on drug absorption differ.

1. Introduction

Efficient and effective drug discovery, achieved with combinatorial chemistry and high-through put screenings, generates numerous lead compounds entering drug/pharmaceutical development processes, where optimal candidates progress through different stages depending on data generated with *in vitro* solubility, permeability, and receptor assays (Stegemann et al., 2007). The failure of *in vitro* models to adequately predict drug/product *in vivo* performance can thus wrongfully terminate further development or it can significantly add to the delays and costs before actual drug marketing. Therefore, it is of outmost importance to mimic bio-relevant conditions in the *in vitro* environment to obtain a correct insight into the actual *in vivo* drug behavior. Furthermore, such an understanding is crucial for the development and interpretation of dissolution tests in generic pharmaceutical drug development prior to entering the bioequivalence trials.

The dissolution methods have attempted to reproduce the environment in the gastrointestinal tract by introducing variations of fasted state simulated intestinal fluid (FaSSIF), and fed state simulated intestinal fluid (FeSSIF) instead of simple water buffers (Vertzoni et al., 2004; Zoeller and Klein, 2007; Jantratid et al., 2008). Sodium taurocholate and lecithin (3 mM taurocholate and 0.75 mM lecithin in FaSSIF; 15 mM taurocholate and 3 mM lecithin in FeSSIF) have been chosen and attempts have been made to replace them by other non-physiological surface acting compounds owing to high cost of pure taurocholate and lecithin and time-consuming buffer preparation process (Zoeller and Klein, 2007). The need for bio-relevant experimental conditions has also been addressed in the *in vitro* permeability measurements. Thus, taurocholate, lecithin and its lipolytic products were included into buffers for permeability experiments (Aungst, 2000; Kataoka et al., 2006; Lind et al., 2007; Patel et al., 2006). Unfortunately, the toxicity of taurocholate towards Caco-2 cell monolayers and rat small intestine was very high (Kataoka et al., 2006; Patel et al., 2006). Caco-2 monolayers lost their integrity and vitality at 10 (Ingels et al., 2002) or at 15 mM (Patel et al., 2006) taurocholate and at 3 mM lecithin (Ingels et al., 2002), while the sensitivity of rat small intestine towards taurocholate was even more pronounced (Patel et al., 2006). Furthermore, it was established that simple ionic

Abbreviations: Ap, apical; P_{app}, apparent permeability coefficient; Bl, basolateral; BL, bile; BCS, Biopharmaceutical Classification System; DMEM, Dulbecco's modified Eagle medium; FaSSIF, fasted state simulated intestinal fluid; FeSSIF, fed state simulated intestinal fluid; F_{abs}, fraction of drug absorbed in humans; c_{free}, free drug concentration; LDH, lactate dehydrogenase; Leci, lecithin; RSE, relative standard error; NaTC, taurocholate; TEER, transepithelial electrical resistance.

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composition of buffers in dissolution testing was inappropriate to sustain an acceptable tissue/cell integrity and vitality, so more complex media were suggested (Hanks balanced salt solution, Leibovitz's L-15 media) (Kataoka et al., 2006; Lind et al., 2007). Osmolarity and pH had to be adjusted too (below 400 mOsm/kg, pH above 6.5) (Kataoka et al., 2006; Lind et al., 2007; Patel et al., 2006).

Although better than water media, simple mixtures of taurocholate and lecithin still do not adequately simulate intra-luminal conditions. Namely, the composition of human bile is far more complex. Taurocholate represents only a minor part of human bile (12–18% of the total bile salts), while glycocholate (26%), glycochenodeoxycholate (20–32%), and glycodeoxycholate (13–16%) constitute other important representatives (Alvaro et al., 1986; Hay et al., 1993; Stolk et al., 1995). The main reason why taurocholate has been favored in the dissolution testing most probably lies in its aggregation number insensitivity to pH changes, ionic strength, and temperature, taurocholate low cost, and the regulatory demand for standardized conditions during the dissolution testing (Alvaro et al., 1986; Stolk et al., 1995). However, a study with two highly lipophilic drugs (ketoconazole and atovaquone) clearly indicated that the use of crude taurocholate (a mixture of ox bile salts) was preferred over the pure taurocholate, because the simulations of the *in vivo* drug performance gave better results with the mixture (Vertzoni et al., 2004). Based on the composition of phospholipids *in vivo*, the *in vitro* use of pure egg phospholipid, lecithin, is not justified either. Namely, the phospholipids in human bile are represented as a combination of different types, which differ in fatty acids esterified to the diglycerides (Hay et al., 1993; Stolk et al., 1995). The blend of phospholipids has been shown to significantly impact the dissolution of highly lipophilic candidates, too (Vertzoni et al., 2004). The mixture of bile salts and phospholipids could thus be a better choice in the assessment of *in vivo* drug performance.

The aim of this work was to evaluate two different biorelevant conditions regarding their impact on the *in vitro* drug permeability. The mixture of sodium taurocholate (NaTC) and lecithin (Leci) or pig bile (BL) were tested on Caco-2 cell monolayers at increasing concentrations and the permeability of 30 drugs was monitored in the absorptive direction. A dialysis of the solutions containing NaTC/Leci or pig bile for all drugs was performed to distinguish between permeation enhancing effects of the surface acting compounds and micellar drug entrapment. Viability and integrity of Caco-2 monolayers were closely monitored. Correlations between *in vivo* fractions of drugs absorbed in humans and *in vitro* permeabilities were also performed. Data were discussed from the view of BCS classification and recently published studies evaluating food effect on the oral drug absorption.

2. Materials and methods

2.1. Materials

Acyclovir, amitryptiline, atenolol, antipyrine, bendroflumethiazide, chlorpheniramine, cimetidine, clomipramine, felodipine, fexofenadine, fluvastatin, furosemide, guanabenz, hydrochlorothiazide, caffeine, lisinopril, loperamide, metoprolol, nadolol, nitrofurantoin, norfloxacin, pindolol, pravastatin, ranitidine, sertraline, sulfasalazine, sumatriptan, terbutaline, timolol and trimetoprim were from Sigma or from Sequoia Research LTD. Pure sodium taurocholate, L-phosphatidyl choline and salt for incubation salines were from Sigma. All chemicals used were of highest grade available. LDH, protein and ATP measurements for cell viability assessment were performed with kits from Promega according to manufacturer instructions.

2.2. Methods

2.2.1. In vitro transport studies across Caco-2 cell monolayers and rat small intestine

Caco-2 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) ACC 169, lot 12 and were grown on Transwell Costar culture inserts with a polycarbonate membrane (diameter 12 mm and pore size 0.4 μm). 50,000 cells/filter membranes were used for seeding and the medium was changed every two days. The medium comprised of Dulbecco's modified Eagle medium (*i.e.* DMEM), fetal bovine serum (20%), L-glutamine (1%) and streptomycin/penicillin (1%). The cells were kept in humidified atmosphere (carbogen (5% CO_2 and 95% of O_2)) at 37 °C. At day 15, transepithelial electrical resistance (TEER) was measured for each filter with Caco-2 cell monolayers. If the TEER values were in the range of 300–400 Ω cm^2, the Caco-2 cell monolayers were used for the subsequent testing of permeability at day 21.

The Caco-2 cells grown on Transwell Costar culture inserts were carefully rinsed with Ringer buffer. 1.5 mL and 0.5 mL of bathing solution (Ringer buffer – apical pH 6.5, basolateral pH 7.4) on basolateral and apical sides of the Caco-2 cell monolayer, respectively, was maintained at 37 °C and continuously oxygenated with carbogen (95% O_2, 5% CO_2) and lightly shaken during experiment. Stock solution of glucose and mannitol (625 mM glucose and 625 mM mannitol) were always added to the basolateral (Bl) and apical (Ap) sides, respectively, to give final 10 mM concentrations of glucose on Bl and mannitol on Ap side. Investigated substances were added to the Ap side at 10–20 μM concentration with or without surface acting compounds (NaTC/Leci or BL). The osmolarity of donor solutions containing NaTC/Leci and BL were checked beforehand (the osmolarity was *ca* 320–370 mOsm/kg). 150 μL samples were withdrawn from the acceptor side every 20 min up to 120 min and replaced by the fresh Ringer buffer containing all necessary ingredients at appropriate concentrations. Only Caco-2 cell monolayers with constant TEER values during the whole experiment were used.

Pig bile was selected because its composition is similar to that of humans (Alvaro et al., 1986; Kobayashi et al., 1998). *In vitro* effect of crude bile on drug permeability has not yet been tested; therefore the increasing content (v/v %) of pig bile was selected based on the literature values; because human gallbladder can store up to 50 mL of bile (Sherwood, 2007), drug could be exposed to 50 mL of bile at the most in fed conditions. Assuming 250 mL as the volume of the ingested fluid one can expect approximately 16.7 v/v% of bile in the intestinal fluid (disregarding the gastric secretions and absorption). *In vitro* fed conditions were thus simulated with 10, 15 and 20 v/v % of crude bile (designated as BL-4, BL-5, and BL-6), while 1, 2, and 5 v/v % (designated as BL-1, BL-2, and BL-3) were selected to represent a constant bile leakage in the fasted state. The combination of NaTC/Leci was tested at 0.2/0.05, 0.5/0.125, 1/0.25, 3/0.75, 7.5/3.25 and 15/7.5 mM (NL-1, NL-2, NL-3, NL-4, NL-5, and NL-6, respectively). NaTC/Leci concentrations in fed state were chosen based on previous studies with Caco-2 cells (*i.e.* NL-4, NL-5 and NL-6 conditions) (Ingels et al., 2002; Lind et al., 2007). Lower NaTC/Leci concentrations were also evaluated. The ratio between NaTC and Leci was kept at 4:1 except for the highest concentration; there, the ratio was 2:1, because taurocholate toxicity can be attenuated with higher amounts of Leci (Lind et al., 2007).

2.2.2. LC–MS/MS analysis

The samples were analyzed by a LC–MS/MS apparatus, which consisted of an Agilent 6460 triple quadrupole mass spectrometer equipped with a JetStream interface and coupled to an Agilent 1290 Infinity UPLC (Agilent Technologies, Santa Clara, USA). The chromatographic separation was performed on a Phenomenex Kinetex 50 × 2.1 mm C18 column (2.6-mm particles), guarded by

a 4 × 2 C18 mm cartridge column (Phenomenex, Torrance, USA). To achieve a better sensitivity and chromatographic peak shape, the compounds of interest were analyzed with two different methods, one for weak bases (mobile phase containing 0.1% formic acid), and the other one for weak acids (mobile phase containing 2 mM ammonium acetate). Therefore, each sample was injected twice: the injection volume was 0.5 and 2.0 μL for weak bases and weak acids, respectively. The column temperature was maintained at 50 °C. The mobile phase A1 was 0.1% formic acid, A2 was 2 mM ammonium acetate, both in Milli-Q water; the mobile phase B1 was 100% acetonitrile and mobile phase B2 was 100% methanol. The gradient A1–B1 and A2–B2 was used for weak acids and weak bases, respectively. The flow rate was 0.6 mL/min and the following linear gradient of the organic modifier was used for both acids and bases (% of B1 or B2): 2%, 10%, 65%, 95%, and 2% in the corresponding time points: 0, 0.8, 1.8, 2.2, 2.3 min, respectively. The total run time was 3.0 min. Mass spectrometry parameters were set as follows: the drying gas temperature: 300 °C, drying gas flow rate: 5 L/min, nebulizer: 45 psi (3.1×10^5 Pa), sheath gas temperature 320 °C, sheath gas flow 11 L/min, capillary entrance voltage 3500 V for positive ions and 4000 V for negative ions, nozzle voltage 1000 V, delta EMV 100 and 200 V for positive and negative ions, respectively. The MRM *m/z* transitions and collision energies are listed in Table 1 in the Supplementary material. The dwell time range was from 20 to 50 ms. Instrument control, data acquisition and quantification were performed by MassHunter Workstation software B.03.01 (Agilent Technologies, Torrance, USA).

The method was partially validated to demonstrate its suitability for the intended study with tested substances of the following validation parameters: linear range, accuracy, precision, autosampler stability and estimation of the limit of detection and quantification (LOD and LOQ). The validation run was deemed acceptable if the accuracy and the precision met the 15% deviation criterion. The robustness was tested by assays of Quality Control samples repeated on each 10 samples in sequence. The matrix effect was estimated by response comparison of standards spiked into the blank incubation medium and into the mobile phase (post spike method). Due to the large number of analytes and also the substantial amount of study results, some of the validation data is shown in Supplementary material (Table 2).

2.2.3. Viability and integrity evaluation

The viability and integrity of Transwell grown Caco-2 cell monolayers were evaluated with TEER measurements every 20 min up to 120 min with the Micropore electrode. Lactate dehydrogenase (LDH), protein release and ATP measurements were performed according to the manufacturer protocols after 2 h exposure of Caco-2 cells to the incubation salines containing increasing concentrations of NaTC/Leci or BL. Cells were considered unharmed, if none of the integrity/vitality markers changed more than 20% compared to the reference values (Lind et al., 2007).

2.2.4. Dynamic dialysis

Based on the assumption that only the free fraction of drug (c_{free}) can permeate through the intestinal lining, a dynamic analysis was performed. The donor solutions were prepared in the same manner as for permeability testing. A cellulose membrane (pore size 3500 g/mol) was immersed in distilled water one day beforehand. On the day of the experiment, the membrane was left for 1 h in the transport medium. The membrane was inserted into dialysis cells. 1 mL of donor solution was added to one side and pure buffer (acceptor solution) to the other. The dialysis cells were sealed to prevent evaporation and left in the incubator at 37 °C, simulating conditions during permeability experiments. The acceptor solution was analyzed by LC/MS/MS after 2-h incubation, before a steady state could be reached – *i.e.* dynamic dialysis method as described

Table 1
The integrity and vitality of Caco-2 monolayers.

Caco-2	NL-1	NL-2	NL-3	NL-4	NL-5	NL-6	BL-1	BL-2	BL-3	BL-4	BL-5	BL-6
TEER	92.2 ± 3.4	101.3 ± 2.5	103.2 ± 2.0	99.1 ± 1.8	106.0 ± 4.9	85.3 ± 8.7	97.9 ± 7.8	94.5 ± 6.1	95.3 ± 3.8	93.3 ± 3.5	91.4 ± 4.8	91.8 ± 7.5
Protein	89.7 ± 6.8	93.3 ± 9.1	84.5 ± 5.3	95.5 ± 5.7	105.4 ± 10.1	104.2 ± 4.7	106.0 ± 3.4	97.4 ± 3.6	96.8 ± 3.4	93.8 ± 2.0	95.2 ± 7.7	94.4 ± 5.2
ATP	107.2 ± 4.2	108.9 ± 2.2	103.0 ± 5.1	104.6 ± 5.0	102.3 ± 6.6	97.3 ± 5.6	103.5 ± 7.2	104.6 ± 4.9	113.1 ± 12.5	86.6 ± 5.1	92.6 ± 4.9	95.2 ± 4.7
LDH	115.2 ± 6.4	127.9 ± 8.0*	125.2 ± 3.7*	128.4 ± 2.9*	146.7 ± 5.1*	187.2 ± 5.4*	103.0 ± 1.5	107.1 ± 1.1	106.0 ± 1.1	104.1 ± 3.2	103.0 ± 2.2	103.6 ± 1.5

The results are presented as means ± SD of 6 measurements. TEER values and the results of protein release and ATP measurement are presented relatively, compared to the reference values (*i.e.* values obtained with Caco-2 cells exposed to incubation buffer without pig bile (BL) or sodium taurocholate/lecithin mixture (NL)). The concentrations tested were 0.2 mM NaTC/0.05 mM Leci (NL-1), 0.5 mM NaTC/0.125 mM Leci (NL-2), 1 mM NaTC/0.25 mM Leci (NL-3), 3 mM NaTC/0.75 mM Leci (NL-4), 7.5 mM NaTC/3.25 mM Leci (NL-5), 15 mM NaTC/7.5 mM Leci (NL-6), 1 v/v% pig bile (BL1), 2 v/v% pig bile (BL2), 5 v/v% pig bile (BL3), 10 v/v% pig bile (BL4), 15 v/v% pig bile (BL5) and 20 v/v% pig bile (BL6).

* Significantly ($p < 0.01$) higher values than the corresponding ones.

Table 2
Physico-chemical and pharmacokinetic drug properties: log *P*, F_{abs}, BCS classification, the mechanism of intestinal membrane transport (active or passive), the importance of first-pass metabolism (FPM) in drug absorption, food effect, the classification of drugs into group A and B based on dialysis results and *in vitro* permeability changes in the presence of NaTC/Leci or bile.

Drug	log *P*	F_{abs} (%)	BCS	Active transport in GIT	FPM[a]	Food effect[b]	Dialysis[c]	P_{app} (NaTC/Leci)[d]	P_{app} (bile)[d]
Acyclovir	−1.56[S1]	20[S2]	III[S3]	Yes (absorptive)[S4]	No[S1]	No/neg[S1]	B	BS	BS
Norfloxacin	−2[S5]	30–40[S6]	IV[S6]	Yes (efflux)[S7]	Yes[S8]	No/neg[S9]	B	BS	BS
Pindolol	−0.92[S5]	89[S10]	I[S10]	No[S11]	Yes[S12]	No[S13]	B	I	BS
Pravastatin	2.45[S5]	34[S14]	III[S3]	Yes (absorptive, efflux)[S15]	Yes[S14]	Neg[S16]	B	I	BS
Sumatriptan	0.79[S5]	62[S17]	III[S18]	Yes (efflux)[S19]	Yes[S20]	No[S21]	B	I	BS
Timolol	−0.53[S5]	60–90[S22,S60]	II[S3]	Yes (efflux)[S23]	Yes[S24]	No[S25]	B	I	BS
Trimetoprim	0.91[S5]	90–100[S26]	III[S3]	Yes (efflux)[S27]	No[S26]	Neg[S28]	B	I	BS
Atenolol	−2.52[S5]	54[S17]	III[S3]	No[S29]	No[S29]	No/neg[S2]	B	I	I
Antipyrine	0.38[S5]	100[S30]	I[S30]	No[S31]	No[S31]	No[S3]	B	I	BS
Cimetidine	0.33[S5]	30–60[S32]	III[S3]	Yes (efflux)[S33]	No[S32]	No[S32]	B	I	BS
Hydrochlorothiazide	−0.1[S5]	55[S30]	III[S3]	No[S30]	No[S30]	Pos[S34]	B	I	BS
Caffeine	−0.16[S5]	100[S35]	I[S3]	No[S35]	No[S35]	No[S36]	B	BS	BS
Nadolol	−2.1[S5]	35[S17]	III[S3]	Yes (efflux)[S37]	No[S38]	Neg[S2]	B	I	BS
Ranitidine	0.79[S5]	61[S17]	III[S39]	Yes (efflux)	No[S38]	No[S40]	B	I	I
Terbutaline	0.34[S5]	60[S41]	III[S41]	No[S41]	Yes[S42]	Neg[S41]	B	I	BS
Amitryptiline	4.92[S5]	90[S43]	I[S44]	Yes (efflux)[S45]	Yes[S46]	No[S46]	A	BS	BS
Bendroflumethiazide	1.95[S5]	>90[S47]	I[S47]	No[S48]	No[S48]	No[S48]	A	BS	BS
Chlorpheniramine	3.39[S5]	80[S2]	I[S3]	No[S49]	No[S49]	Unknown[S2]	A	BS	BS
Clomipramine	5.19[S5]	80[S50]	III[S51]	Yes (efflux)[S50]	Yes[S52]	No[S53]	A	BS	BS
Furosemide	−0.8[S5]	50[S17]	III[S3]	Yes (efflux)[S54]	Yes[S55]	No/neg[S56,S57]	A	BS	BS
Guanabenz	2.25[S5]	75[S58]	II[S59]	No[S60]	Yes[S61]	Unknown	A	BS	BS
Metoprolol	−0.82/1.72[S5]	96[S30]	I[S3]	No[S3]	Yes[S62]	No/pos[S63]	A	NC	NC
Sulfasalazine	3.94[S5]	13[S30]	IV[S64]	Yes (efflux)[S64]	No[S65]	Unknown	A	BS	BS
Felodipine	3.8[S5]	45–80[S66]	II[S67]	No[S3]	Yes[S68]	No/pos[S67]	A	BS	BS
Fexofenadine	2.81[S5]	1–3[S69]	III[S3]	Yes (absorptive, efflux)[S70]	Yes[S71]	No/neg[S72]	A	I	BS
Fluvastatin	4.2[S5]	98[S17]	I[S73]	Yes (efflux)[S74]	Yes[S75]	No[S76]	A	BS	BS
Lizinopril	−3.4[S5]	25[S77]	III[S3]	Yes (absorptive)[S78]	No[S79]	No[S79]	A	I	BS
Loperamide	4.44[S5]	65–100[S80]	II[S80]	Yes (efflux)[S81]	Yes[S82]	No[S82]	A	BS	BS
Nitrofurantoin	−0.47[S5]	100[S83]	IV[S84]	Yes (efflux)[S85]	No[S83]	Pos[S86]	A	BS	BS
Sertraline	5.15[S5]	44[S87]	II[S88]	Yes (efflux)[S89]	Yes[S90]	No/pos[S90]	A	BS	BS

[a] *FPM*: importance of first-pass metabolism on drug oral bioavailability according to the literature.

[b] *Food effect*: the impact of food on the extent of absorption – AUC (positive: more than 25% AUC increase, negative: more than 20% AUC decrease, no: AUC inside the 80–125% of the confidence interval of AUC in fasted state).

[c] *Dialysis*: c_{free} was significantly lowered by NaTC/Leci and bile (A for group A) or only by bile (B for group B). [S1–90]: references for data in Table 2 provided in the Supplementary material.

[d] P_{app} (NaTC/Leci), P_{app}(Bile): the trend of *in vitro* permeability changes determined in the presence of increasing NaTC/Leci and bile concentrations. BS–bell shaped trend of P_{app} changes (initial P_{app} increase followed by a decrease); I – P_{app} increases with increasing concentration of surface acting compounds, NC – no trend of P_{app} changes.

by Yano et al. (2010). The non-specific drug binding to lab ware was also assessed by subsequent exposure of the same test solution to multiple vessels and pipette tips. The % of free drug was calculated according to Eq. (1).

$$\%_{free} = \frac{c_{free}}{c_{tot} - c_{free}} \times 100 \quad (1)$$

where c_{free} is the concentration of free drug in the acceptor solutions and c_{tot} represents the donor concentration of the drug. The results are presented as means ± SD of five measurements.

2.2.5. Data analysis

The apparent permeability coefficient (P_{app}) of drugs was calculated according to Eq. (2)

$$P_{app} = \frac{dc}{dt}\frac{V}{c_0 A} \quad (2)$$

where dc/dt represents changes in concentration/mass of the examined substance in the acceptor compartment per unit time under steady state conditions, V is the volume of the acceptor compartment, A the exposed surface area (1.13 cm^2 for Caco-2 cell monolayers) and c_0 the initial concentration of the drug.

Results in tables and figures are presented as means ± SD of at 4 measurements. Data were evaluated statistically using SPSS 17.0 (SPSS Inc., Chicago, IL). F-test for testing the equality of variances and 2-tailed Student t-test were used to compare determined permeabilities to the corresponding reference values. Significance level was set to 0.01.

Correlation between human absorption (F_{abs}) and apparent permeability coefficient was described using Hill equation (Saitoh et al., 2004), Eq. (3):

$$F_{abs} = \frac{P_{app}^{\gamma}}{P_{app}^{\gamma} + P_{app\,50}^{\lambda}} \quad (3)$$

where γ is the Hill's coefficient and $P_{app\,50}$ is the permeability, which corresponds to 50% of F_{abs}. The parameters of Hill equation and its uncertainty were estimated using a nonlinear regression analysis in SPSS 17.0. The uncertainty of parameters estimates was reported as relative standard error (RSE). The coefficient of determination (R^2) was also calculated.

3. Results

3.1. The integrity and vitality of Caco-2 cell monolayers

The integrity and vitality of Caco-2 cell monolayers were assessed by measuring TEER, the release of LDH and proteins into the incubation media and by measuring intracellular ATP concentration, which were then compared to the reference values and expressed relatively (Table 1). TEER values progressively decreased during experiments with surface acting compounds (data not shown) but this decrease was not significant. The ratios between the last measured TEER values (determined at 120 min) and the initial TEER values (*i.e.* before the experiments) were not significantly lower; they were all above 90% (Table 1). Similarly, the release of proteins and ATP concentrations did not change significantly. However, LDH release increased significantly in the presence of NaTC and Leci at all applied concentrations (NL-2, NL-3, NL-4, NL-5) except at the lowest one (NL-1). The highest concentration of NaTC and Leci (NL-6: 15 mM and 7.5 mM) was not further tested for its impact on drug permeability because of significant vitality/integrity decline. Bile did not significantly influence LDH release (Table 1).

3.2. Dialysis and the free fraction of drugs in donor solutions containing NaTC/Leci and pig bile

The results of the dialysis are shown in Figures 1–30 provided in the Supplementary material. The free drug concentration (c_{free}) in donor solutions was considered significantly lowered, if it was reduced below 80% of the initial value. Regarding the results, we observed that for some compounds, c_{free} was significantly affected by the presence of both, bile and NaTC/Leci, so they were then categorized into the group A (amitryptiline, bendroflumethiazide, chlorpheniramine, clomipramine, felodipine, fexofenadine, fluvastatin, furosemide, guanabenz, lisinopril, loperamide, metoprolol, nitrofurantoin, sertraline and sulfasalazine). For the other drugs, c_{free} was lowered only in the presence of bile and were thus classified in group B (acyclovir, atenolol, antipyrine, cimetidine, hydrochlorothiazide, caffeine, nadolol, norfloxacin, pindolol, pravastatin, ranitidine, sumatriptan, terbutaline, timolol and trimetoprim).

In group A, a significant decline of c_{free} was noticed at low percentages of bile and at lower concentrations of NaTC/Leci (at 1 v/v% of bile for amitryptiline, felodipine, fluvastatin and sulfasalazine; at 2 v/v% for bendroflumethiazide, chlorpheniramine, clomipramine, guanabenz, loperamide, sertraline; at 5 v/v% for fexofenadine and from 1 mM NaTC/0.25 mM Leci on for all drugs). These substances were more hydrophobic drugs with positive log P values (Table 2). On the other hand, for the more hydrophilic drugs in group A, such as furosemide, lisinopril, metoprolol and nitrofurantoin with negative log P values (Table 2), a significant decline of c_{free} was observed at the highest percentages of pig bile (15 or 20 v/v%) and at the highest NaTC/Leci concentrations (7.5 mM/3.75 mM).

In the group B, c_{free} was significantly lowered at 5 v/v% of bile for acyclovir, norfloxacin, pindolol, pravastatin, sumatriptan, timolol and trimetoprim and at 15% v/v% for atenolol, antipyrine, cimetidine, hydrochlorothiazide, caffeine, nadolol, ranitidine, and terbutaline (Table 2).

3.3. The in vitro drug permeabilities through Caco-2 cell monolayers

In the presence of increasing NaTC/Leci and bile concentrations, three types of *in vitro* permeability changes were observed. In the first case, a bell shaped relationship between *in vitro* permeabilities and increasing concentrations of NaTC/Leci or bile was noticed; the permeabilities initially (in)significantly increased, reaching a plateau value, which was then followed by a permeability decrease, so that the permeabilities, measured at the highest concentrations of NaTC/Leci or bile were either identical or already significantly lower than those of the reference (Table 2 – designated as BS). In some cases, the permeabilities gradually increased with increasing concentrations of NaTC/Leci or bile and remained elevated (Table 2 – designated as I). In the third case, the permeabilities did not show any tendencies to change in the presence of NaTC/Leci and bile (Table 2 – designated as NC).

In the group B (compounds whose c_{free} were significantly lowered only by bile), bell shaped permeability changes in the presence of bile were observed for all tested candidates except for atenolol and ranitidine. NaTC/Leci, the excipients which did not affect c_{free}, induced permeability increase with increasing concentrations almost for all tested drugs except for acyclovir, norfloxacin and caffeine, where a bell shaped relationship was determined. In the group A (bile and NaTC/Leci significantly lowered c_{free}), permeabilities followed a bell shaped curve in all cases except for metoprolol, where no change in P_{app} was observed, while for fexofenadine and lisinopril the presence of NaTC/Leci caused an increase in P_{app} (Table 2).

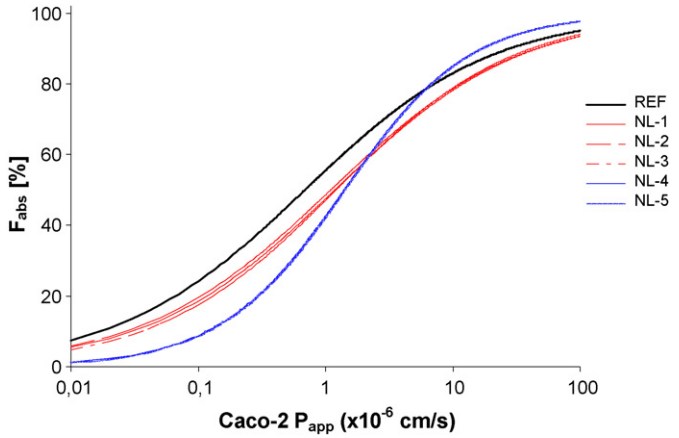

Fig. 1. The correlation of fractions of drugs absorbed (F_{abs}) in humans with Caco-2 absorptive drug permeabilities, determined in Ringer buffer (REF) and in the presence of NaTC and Leci at molar ratios of 0.2/0.05, 0.5/0.125, 1/0.25, 3/0.75, 7.5/3.25 and 15/7.5 mM for NL-1, NL-2, NL-3, NL-4, NL-5.

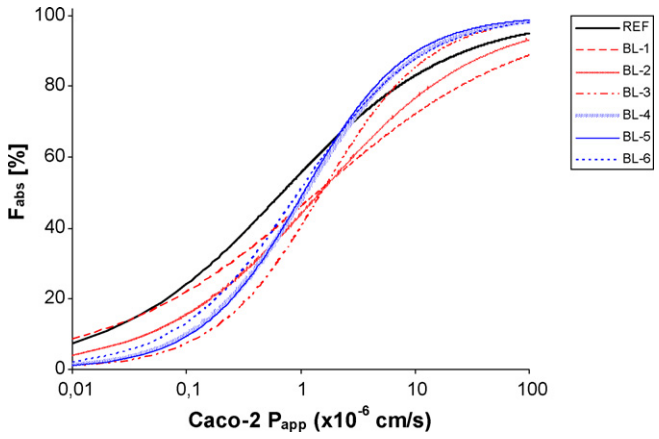

Fig. 2. The correlation of fractions of drugs absorbed (F_{abs}) in humans with Caco-2 absorptive drug permeabilities, determined in Ringer buffer and in the presence of crude pig bile. The bile content was 1, 2, 5, 10, 15, and 20 v/v%, in BL-1, BL-2, BL-3, BL-4, BL-5, and BL-6, respectively.

According to Rege et al. (2001), at least 300% change of *in vitro* P_{app} values through Caco-2 cell monolayers is needed to expect a significant *in vivo* permeability change. A 3-time permeability decrease in the presence of 10, 15 and 20 v/v% of bile was observed *in vitro* for amitryptiline, clomipramine, fluvastatin, guanabenz, loperamide and sertraline. A 3-time P_{app} decrease was observed also for amitryptiline, clomipramine and sertraline in the presence of NaTC/Leci (at NL-4, NL-5, and NL-6).

An identical permeability increase was determined for furosemide and lisinopril in the presence of bile and NaTC/Leci and for acyclovir, atenolol, hydrochlorothiazide, nadolol, pravastatin, and terbutaline at the highest NaTC/Leci concentrations (NL-4 and/or NL-5). 3-times higher P_{app} values in the presence of bile were observed for clomipramine, fexofenadine and sulfasalazine at the lowest bile content tested (*i.e.* B-1: 1 v/v%), for acyclovir and pravastatin at all bile concentrations, and for atenolol, hydrochlorothiazide, nadolol, sumatriptan and terbutaline at the highest tested bile concentrations.

3.4. The correlations between the in vitro P_{app} and fractions of drug dose absorbed (F_{abs})

In vitro determined P_{app} values were fitted to F_{abs} according to Eq. (3). F_{abs} values were taken from the literature (Table 2). The correlations indicated a curve shift to the right when surface acting compounds were present (Figs. 1 and 2). Estimates of the Hill equation parameters, relative standard error (RSE), and a coefficient of determination for data fit (R^2) are given in Table 3. In all cases the RSE values were below 50% and the R^2 values were relatively high. The curves obtained with simulations for NaTC/Leci at 0.2/0.05 (NL-1), 0.5/0.125 (NL-2), 1/0.25 mM (NL-3) and for NaTC/Leci at 3/0.75 (NL-4) and 7.5/3.25 (NL-5) mM exerted similar shapes (the curves for NL-1–NL-3 are overlaid one on top of the other – while the curves for NL-4 and NL-5 were almost identical but differed significantly from the former ones). In the case of bile (Fig. 2) the overlay/similarity between simulated curves was less evident.

Table 3
Estimates of parameters of Hill equation and coefficient of determination (R^2) for data fit obtained with Caco-2 cell monolayers.

Caco-2 cells	$P_{app\ 50}$ (% RSE)	Hill coeff. (% RSE)	R^2
Ref	0.690 (29.5)	0.593 (21.4)	0.584
BL-1	1.411 (43.4)	0.482 (27.6)	0.412
BL-2	1.477 (29.4)	0.624 (23.5)	0.526
BL-3	1.536 (17.5)	0.931 (21.9)	0.62
BL-4	1.095 (17.1)	0.912 (19.6)	0.672
BL-5	1.060 (16.3)	0.959 (20.7)	0.667
BL-6	0.966 (21.2)	0.833 (23.4)	0.576
NL-1	1.184 (30.1)	0.597 (22.0)	0.563
NL-2	1.110 (30.6)	0.591 (22.3)	0.555
NL-3	1.211 (28.3)	0.622 (21.8)	0.573
NL-4	1.397 (17.1)	0.884 (18.7)	0.701
NL-5	1.428 (19.4)	0.884 (22.7)	0.592

RSE: relative standard error.

4. Discussion

4.1. The impact of surface acting compounds on the viability and integrity

Sustaining an acceptable integrity and vitality of cell cultures during the *in vitro* permeability measurements is a necessary prerequisite for reliable predictions of *in vivo* absorption. This is especially important when surface acting compounds are included in the transport media, because they can readily distribute into the lipid bilayer and trigger a detergent action (Klinkspoor et al., 1999). The mixture of taurocholate and lecithin promotes drug absorption by compromising the structure and viscosity of mucus, by inhibiting metabolizing enzymes and eliminative transporters, and by increasing the paracellular diffusion (Hussain et al., 2004; Maeney and O'Driscoll, 2000; Poelma et al., 1991). All these drug penetration enhancing effects are closely associated with NaTC/Leci ability to solubilize membrane proteins and phospholipids, which eventually leads to cell desquamation, erosion and death (Hussain et al., 2004; Waller et al., 1988). *In vivo* drug absorption can also be improved with bile salts (*i.e.* fed state) which enhance the rate of drug dissolution, solubility and membrane transport (Schwarz et al., 1996). Due to the toxic nature of these adjuvants (Klinkspoor et al., 1999; Lind et al., 2007), the viability and integrity in this study were closely monitored. The use of at least two independent vitality/integrity parameters has been suggested to assess cell damage evoked by surface acting compounds (Lind et al., 2007) – electrophysiology and the release of cellular markers (*i.e.* protein, LDH, ATP).

Caco-2 cells were practically insensitive to the bile salts, while NaTC/Leci combinations increased the release of LDH significantly (Table 1). Although other controlled parameters did not indicate significant membrane damage, local membrane injuries were most probably triggered by NaTC/Leci. LDH release is the most sensitive parameter to assess compound toxicity (Lind et al., 2007). Namely, damaged cells (significant damages to the cell membrane) can remain attached to the filter during experiments, making the

measurements of cell protein release less reliable. Similarly, TEER values are believed to decrease significantly on the onset of irreversible damages (Lind et al., 2007). Owing to the absence of mucus on the apical side of Caco-2 cells, they are much more sensitive to detergents than cells *in vivo* (Maeney and O'Driscoll, 1999). However, *in vivo* exposure of the absorptive cells to bile also leads to local, but reversible membrane damage, accompanied by significantly increased LDH release (Klinkspoor et al., 1999). However, contrary to the *in vitro* conditions, cells *in vivo* can recuperate or can be replaced quite rapidly by the new ones emerging from the undamaged crypts (Aungst, 2000; Waller et al., 1988). The reconstitution of damaged Caco-2 cells is most probably not equally fast *in vitro* as it is in case of enterocytes *in vivo*. Significantly increased LDH release detected in this study thus demonstrates environmental insult of NaTC/Leci towards Caco-2 cells, similar to the *in vivo* situation. Since other parameters (TEER, protein release, ATP) did not indicate an irreversible cell damage, the cells were utilized for the permeability experiments but NaTC/Leci was added to the transport media at concentrations below the ones in FeSSIF (15 mM NaTC and 7.5 mM Leci) and the ratio between NaTC and Leci was kept at 1:2 for NL-5 (Patel et al., 2006). Crude bile was not cytotoxic, since none of the monitored parameters changed significantly. The release of LDH was even smaller than in unexposed Caco-2 cells, which is in accordance with the results published by Klinkspoor et al. (1999), where a decreased LDH release from LS174T human epithelial colon cell line in the presence of low concentration of bile was observed.

4.2. The in vitro drug permeability and entrapment into mixed micelles

Above critical micellar concentration bile and NaTC/Leci organize into spherically shaped mixed micelles. Depending on the physicochemical drug characteristics (solubility, log *P*, p*K*a, polar surface area, total surface area, the number of H-donor/acceptor groups, steric influences, electrostatic and dipole interactions), interactions between drugs and micelles enable faster drug dissolution, solubility and influence drug absorption (Palm et al., 1997; Schwarz et al., 1996). Surface acting compounds can exert dual impact on the absorption. They can increase drug absorption by incorporating into the cell membrane (permeability enhancers), by an increased collisional drug transfer from micelles to glycocalyx and by reducing UWL effect (Lind et al., 2007). However, drug interactions with mixed micelles also lower their c_{free}, which could significantly decrease drug permeability and absorption (Jones et al., 2006; Poelma et al., 1991).

NaTC/Leci and bile salts were used to evaluate their impact on permeability and micellar drug entrapment. Since human bile is impossible to obtain, pig bile was selected because of its high resemblance regarding the composition of phospholipids and bile salts (Alvaro et al., 1986; Coleman et al., 1979; Kobayashi et al., 1998; Lapidus and Einarsson, 1998; Legrand-Defretin et al., 1991). Increasing concentrations were tested to address the question whether lower amounts could be used *in vitro* to predict their effects on drug absorption on the one hand and to assure acceptable cell viability on the other. The reasons for selection of NaTC/Leci and bile concentrations are mentioned in Section 2.

The results indicated that while c_{free} for one group of tested drugs was significantly lowered by both types of surface acting compounds (*i.e.* group A), only bile had a significant impact on c_{free} of the other (*i.e.* group B). Since both types of additives can act as permeability enhancers and as colloid structures decreasing drug's c_{free}, the relationship between determined *in vitro* P_{app} values and increasing concentrations of NaTC/Leci and bile (for group A) or just bile (for group B) were bell shaped. Initially – at lower surfactant concentrations, the drug incorporation into mixed micelles was most probably not so profound. Therefore, the permeability enhancing effect prevailed and one could observe a gradual permeability increase. However, eventually, drug entrapment into micelles became significant, causing c_{free} and P_{app} to decrease. NaTC/Leci did not lower c_{free} for drugs in group B, therefore the P_{app} increase with increasing concentrations of NaTC/Leci was induced most probably only by the penetration enhancing effect. The exceptions to these observations (lisinopril, fexofenadine, caffeine, acyclovir and norfloxacin) represent the borderline drugs, whose c_{free} at the highest NaTC/Leci or bile concentrations were slightly below or above the critical 80% and could thus easily be re-classified into the opposite groups.

Based on supplementary figures, the ratios between c_{free} and c_{tot} (*i.e.* % free fraction) were usually lower than the ratios between P_{app} determined in the presence of SIFs and reference P_{app} values (*i.e.* while the % of free fractions were below 10% for some drugs, the ratios between permeabilities were not, or were even significantly higher – more than 300%). This was observed also by Yano et al. (2010), who studied troglitazone transport through Caco-2 cells in the presence of NaTC. By comparing the above mentioned ratios, an additional absorption pathway in a particulated form (*i.e.* micelles) not dependent on troglitazone c_{free} was proposed (Yano et al., 2010). The c_{free} in this study were also lower than the corresponding permeability ratios, which were significantly higher than 1 for some drugs. This indicates that an additional transport pathway for the entrapped drugs through Caco-2 cells could exist also for some substances presented in this paper (Yano et al., 2010).

4.3. The correlation of in vivo fraction of drug absorbed with in vitro permeabilities

Fitting the *in vitro* P_{app} with F_{abs} values indicated that curves obtained for NaTC/Leci at low (NL-1, NL-2 and NL-3) and high (NL-4 and NL-5) concentrations had a low and a high effect on permeability, respectively. The differences within the low and the high effect curves were minimal. Therefore, the impact of NaTC/Leci on drug permeability could be monitored at 0.2/0.05 mM for fasted and at 3/0.75 mM for fed conditions to minimize cell damage (Fig. 1). The overlay between simulated curves obtained with low (*i.e.* 1, 2, and 5 v/v%) or with high (10, 15, and 20 v/v%) bile was less evident. However, to simulate fed conditions *in vitro*, one does not need to use extremely high bile concentrations (*i.e.* 20 v/v%), because 10 v/v% of crude bile would give about the same information regarding P_{app} changes quantitatively.

In all simulations with either bile or NaTC/Leci curves shifted to the right. The drug permeability through Caco-2 monolayers would thus increase, but the final effect on the F_{abs} would not significantly improve for the set of the tested drugs in the presence of bile nor NaTC/Leci. This is in accordance with the literature data of food effects on drug bioavailability for 30 candidates studied (Table 2). For 30 candidates tested, the F_{abs} does not change for the majority (no F_{bas} change in 13 cases, negative for 4 drugs, positive for 2, negative or none for 5, and positive or none for 3, no reports for 3 – Table 2); therefore, our model correctly simulated the *in vivo* situation in fed state.

Attempts have been made to explain and simulate drug bioavailability in fed conditions with BCS/BDDCS classification (Wu and Benet, 2005), GUT framework (Sugano et al., 2010), logistic regression (Gu et al., 2007) and by correlating different physicochemical drug properties with the P_{app} and/or F_{abs} (Singh, 2005). However, major contributing factors and the details of food effect regarding drug absorption still remain unexplained, because there are so many factors acting in tandem (drug solubility, dissolution rate, permeability, stability and physiological adjustments in digestive tract to the presence of food), which cannot all be considered simultaneously. In general, drug absorption from intestine could

either be classified as permeability-limited, dissolution rate limited and/or solubility limited (Sugano et al., 2010). In the last two scenarios, positive or slightly positive food effect on drug absorption is anticipated (Sugano et al., 2010). In our study dissolved compounds were applied, therefore the impact of bile and NaTC/Leci on solubility and dissolution was avoided and only membrane permeability (*i.e.* permeability limited absorption) could be monitored as a function of drug incorporation into mixed micelles on one hand and penetration enhancing effects on the other. Mixed micelles will lower the c_{free} in fed state, which could result in negative or no food effect on the absorption (Sugano et al., 2010). According to BCS the effect of food on the AUC will be negligible for BCS I compounds, but it will be positive for BCS II (they exert dissolution-limited absorption and are substrates for efflux transporters) and negative for BCS III compounds (because of the inhibition of absorptive transporters, the inhibition of efflux transporters is usually less important). The impact on BCS IV drugs is complex and usually not defined (Kawai et al., 2011; Wu and Benet, 2005). The BCS assumptions regarding the food influence on drug absorption are based on the local drug concentrations, achieved in the gut after the intake of immediate release formulation, and drug's intestinal pre-systemic metabolism (*i.e.* drug preferences for absorptive and/or secretory transporters and intestinal enzymes) (Wu and Benet, 2005).

The absorption of highly permeable BCS I and II drugs usually proceeds in the proximal parts of the small intestine when administered in immediate release formulation (Wu and Benet, 2005; Sugano et al., 2010). There the impact of drug distribution into mixed micelles and consequently decreased c_{free} will be less important than potential effects of surfactants (*i.e.* NaTC/Leci or bile) on the membrane, mucus, transporters and tight junctions (Sugano et al., 2010), because in this early phase of drug release and dissolution, the total amount of dissolved drug in fed state exceeds the one in the fasted (Kawai et al., 2011). This was also observed in this study, where the permeability of the majority of tested BCS I and II candidates remained unchanged (insignificant P_{app} changes – Table 2, Supplementary material). However for some substances from both classes permeabilities significantly decreased owing to the profound decrease in c_{free} values, which represents the so-called middle-to-late dissolution phase (Sugano et al., 2010). Since we experimented with solutions, prepared more than 30 min prior to the actual permeability experiment, the drugs must have already distributed into the colloidal micellar structures, profoundly lowering c_{free} values, which reflected as permeability decrease. For these identified candidates, the extent of drug absorption in fed *versus* fasted conditions could yield important differences.

The majority of the tested drugs in this study represent BCS III of highly soluble but poorly permeable compounds. Their absorption through enterocytes proceeds by absorptive and secretory transporters, which can be modulated by food components – negative food effect (Wu and Benet, 2005). Negative food effect has also been suggested by Kawai et al. (2011) owing to decreased c_{free}. However, these substances are preferentially hydrophilic and could benefit from the penetration enhancing effects and inhibition of efflux transporters by NaTC/Leci and bile as evident for some substances in this study (*i.e.* acyclovir, pravastatin, atenolol, hydrochlorothiazide, nadolol, terbutaline, furosemide, and lisinopril). *In vivo* positive food effect has been noticed only for hydrochlorthiazide, while for other compounds no or negative effects have been published (Table 2). The absorption of BCS III drugs is permeability-limited, therefore the absorption proceeds not only in the upper, but also in the lower parts of the small intestine, where the amount of unbound drug is significantly lowered in fed conditions (Kawai et al., 2011). Based on this, *in vivo* drug binding to micelles and lower passive diffusion most probably prevailed over inhibited efflux transporters noticed in this *in vitro* study. However, with NaTC/Leci, these drugs did not incorporate into the mixed micelles, and at the same time, the permeability significantly increased. Due to the differences between NaTC/Leci and bile, NaTC and Leci could be incorporated into the formulations with acyclovir, pravastatin, atenolol, hydrochlorthiazide, nadolol, terbutaline, furosemide and lisinopril to increase their absorption, if the release of NaTC/Leci would be sufficient at the site of the absorption to ensure effective NaTC/Leci amounts over the absorptive area even after spreading and dilution by the intestinal content.

This study did not show any permeability improvement for BCS IV tested candidates (norfloxacin, sulfasalazine and nitrofurantoin Table 2) by none of the applied surfactants.

5. Conclusion

The evaluation of drug permeability *in vitro* to address the *in vivo* drug absorption demands the use of surface acting compounds. The combination of NaTC/Leci has been used to mimic the *in vivo* conditions, but the toxicity of these excipients often precludes their use. Therefore, increasing concentrations of NaTC/Leci were tested in this study while closely monitoring the Caco-2 viability and integrity. The correlation of the measured permeabilities with the F_{abs}, showed that fasted and fed conditions could be simulated with lower amounts of both adjuvants to prevent the cell damage. Since NaTC/Leci does not copy the exact composition of intraluminal content, pig bile was also tested. Based on the dialysis results and *in vitro* permeabilities, replacing NaTC/Leci with bile does not necessarily yield the same results, because the interaction of drugs with colloids and the mechanisms, exerted by bile salt or NaTC/Leci on membrane properties differ significantly. Therefore, a careful selection of bio-relevant transport media based on the dialysis is needed before the permeability assays are performed. Although the distribution of drugs into micelles is believed to decrease c_{free} available for absorption, this was not the case in the presented paper. Although significantly lower c_{free} was determined for some substances, the permeability significantly increased, indicating permeability enhancing effect of these excipients and that an additional, not yet identified transport pathway for drugs in the micelles could exist.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2012.03.015.

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