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Use of simulated intestinal fluid for Caco-2 permeability assay of lipophilic drugs

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ABSTRACT

The most commonly used method to assess intestinal permeability is the measurement of compound flux across a Caco-2 cells monolayer by using Hanks balanced salt solution (HBSS)-like buffers. Nevertheless, lipophilic acid drugs are poorly or not at all soluble in these types of buffers and their adsorption on the transwell plate is commonly observed. To reduce adsorption and increase solubility, permeability assays need to be developed in conditions other than classic conditions for lipophilic compounds. The best model to increase recovery of lipophilic compounds was determined as fasted state simulated intestinal fluid (FaSSIF) in the apical compartment and HBSS with 1% bovine serum albumin (BSA) in basolateral compartment. This model allows a correlation between absorption on Caco-2 cells and absorbed fraction in humans. For 35 compounds, only 2 outliers were observed in the Caco-2 assay using the FaSSIF model. These two outliers were the same outlier compounds as those observed with a classic Caco-2 method. Furthermore, a permeability assay of Pgp substrates evidenced efflux transport in both models and addition of a Pgp inhibitor suppressed Pgp efflux transport. FaSSIF in the apical compartment and HBSS with 1% BSA in the basolateral compartment is the model of choice to predict *in vivo* absorption for lipophilic acid drugs.

1. Introduction

Oral administration of therapeutics, when appropriate, is the preferred route for drug delivery. Consequently, the transport of drugs across the intestinal epithelial cell barrier is a major determinant of *in vivo* bioavailability. Various *in vivo* and *in vitro* methods exist to measure a compound's bioavailability (Audus et al., 1990; Artursson and Borchardt, 1997; Hidalgo, 2001). The most commonly used well-established method to assess potential intestinal permeability is the measurement of compound flux across a monolayer of Caco-2 cells. Caco-2, established by Jorgen Fogh and co-workers (Fogh and Trempe, 1975; Fogh et al., 1977) is effectively a cell line that exhibits morphological and functional characteristics of small intestinal cells. Caco-2 cells form tight junctions and develop microvilli, express brush border enzymes, growth factor receptors and major drug-metabolising enzymes (Pinto et al., 1983; Grasset et al., 1984; Hidalgo et al., 1989; Prueksaritanont et al., 1996; Delie and Rubas, 1997) except CYP3A4 (Gan et al., 1996; Delie and Rubas, 1997; Hu et al., 1999; Borlak and Zwadlo, 2005). Furthermore, Caco-2 cells possess an array of transporters found in the absorptive intestinal cells, like P-glycoprotein (Pgp) and others like multidrug resistance-associated proteins, organic anion transporter proteins, organic anion transporters, etc. (Karlsson et al., 1993; Delie and Rubas, 1997; Stratmann et al., 1997; Borlak and Zwadlo, 2005; Maubon et al., 2007). It has been generally accepted that compounds with a good permeability in Caco-2 assays have good human intestinal absorption (Artursson, 1991; Artursson and Karlsson, 1991).

Permeability assay on Caco-2 cells is generally performed with Hanks balanced salt solution (HBSS)-like buffers. Nevertheless, medicinal chemistry in pharmaceutical companies generates more and more drugs which have high lipophilicity (Lipinski et al., 1997). These lipophilic drugs are generally not or only poorly soluble in HBSS-like buffers. Furthermore, non-specific adsorption of these

Abbreviations: Caco-2, human Caucasian colon adenocarcinoma; Fabs, absorbed fraction in human; FaSSIF, fasted state simulated intestinal fluid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HBSS, Hanks' balanced salt solution; LC–MS/MS, liquid chromatography–tandem mass spectrometry; Papp, apparent permeability; MES, 2-*N*-Morpholinoethanesulfonic acid monohydrate; Pgp, P-glycoprotein.

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drugs on Caco-2 transwell plates occurs to a large extent when HBSS-like buffers are used. Therefore, for these kinds of compounds, a specific Caco-2 permeability assay has to be developed to improve drug recovery and permeability assessment. Different conditions to improve Caco-2 permeability assays have been described by various authors (for example, using different solubilizers: Anderberg et al., 1992; Takahashi et al., 2002; for the Ingels and Augustijns review, 2003). Addition of proteins, like bovine serum albumin (BSA) in the basolateral compartment have been proved to increase the solubility and reduce adsorption on the plate during Caco-2 permeability assay, thus improving the recovery and the apparent permeability results (Aungst et al., 2000; Yamashita et al., 2000; Krishna et al., 2001). For example, Krishna et al. have shown that inclusion of up to 4% BSA in the basolateral side provides necessary absorptive driving force similar to *in vivo* conditions, improving both recovery and apparent permeability (Papp) of two highly lipophilic and highly protein-bound compounds from Schering as well as those of progesterone, a reference for highly lipophilic and protein-bound compounds. On the other hand, the recovery and Papp of mannitol (high recovery, low permeability) and propranolol (high recovery, high permeability) remain unaffected. Furthermore, Ingels et al. (2002, 2004) have shown that fasted state simulated intestinal fluid (FaSSIF) medium is compatible with the Caco-2 model for at least 2 h, and the use of simulated intestinal fluids such as apical buffer improve permeability assay results by increasing solubility and recovery for poor water soluble drugs. However, sodium taurocholate, present in FaSSIF, has a Pgp inhibitory effect that decreases the permeability ratio between both directions compared to the classic method. Nevertheless, bile salts, including sodium taurocholate, are also present in physiological conditions, as cited by Ingels et al. (2004): "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". Furthermore, Patel et al. (2006) have shown that microvilli generally maintain a normal distribution with FaSSIF buffer in apical compartment. Trans epithelial electric resistance (TEER) is maintained for over 4H and mannitol permeability is equivalent to that in Caco-2 permeability assay with HBSS.

Since bile salts are present in the intestinal lumen and proteins in the blood, permeability and recovery results for different compounds, including highly lipophilic drugs, were compared between different conditions: replacement of HBSS by FaSSIF in apical buffer or addition of BSA in basolateral compartment or the association of both. Furthermore, after selecting the best model for lipophilic drugs, 35 known molecules were then tested with a "classic" method using HBSS-like buffers and the new chosen model and a correlation between the percentage of absorbed fraction in human (Fabs) and permeability on Caco-2 cells (Papp) was determined for both models.

2. Material and methods

2.1. Material

Caco-2 cells (passage # 4) were obtained from European Collection of Cell Culture (ECACC, UK). Modified Eagle's medium, non-essential amino acids and antibiotics, fetal bovine serum and trypsin–EDTA were purchased from Gibco (Invitrogen, Cergy Pontoise, France). Hanks' balanced salt solution with or without phenol red, 2-*N*-morpholinoethanesulfonic acid monohydrate (MES), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), BSA, lecithin, L-alpha-phosphatidylcholine, taurocholic acid, KCl, atenolol, digoxin, and all other reagents and 35 used compounds in permeability assay were purchased from Sigma (Saint Quentin Fallavier, France). Thirty-three used compounds were synthesised at laboratories Fournier. Multiscreen Caco 96-well microplates were purchased from Millipore (Saint-Quentin en Yvelines, France). The automation platform was a Caliper LifeSciences staccato made up of a liquid handler: CaliperLS Sciclone ALH 500 equipped with a High Volume 96-tip Head, Z8 and Gripper, an incubator: Kendro CO_2 Cytomat 6000, a plate handler and stacker: CaliperLS Twister II and a scheduler: Clara software.

2.2. Cell culture

Caco-2 cells were routinely maintained in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acid, 100 U/ml penicillin-G and 100 μg/ml streptomycin. Cells were incubated at 37 °C in 5% CO_2. Medium was changed every 2 days. For permeability assay, cells were used between passage 35 and 50.

2.3. Drug permeability assay

Cells were seeded at 10^5 cells/cm^2 apically in the 96-well MultiScreen Caco-2 plates and then incubated at 37 °C in 5% CO_2 for 21 days for differentiation. Medium was changed every 2 days in both compartments. Permeability assay was performed on Caliper LifeSciences staccato. After pre-incubation at 37 °C for 20 min and removal of medium, for apical to basolateral transport (A–B), compounds (10 μM) in apical medium [HBSS buffer with MES 2.5 mM, pH 6.5 or FaSSIF buffer (3.9 g/l KH2PO4, 7.7 g/l KCl, 0.3 mM Na-taurocholate and 0.075 mM egg lecithin pH 6.5)] containing atenolol, the membrane integrity marker, were added apically (75 μl) and basolateral medium (HBSS buffer containing HEPES 5 mM, pH 7.5 with or without BSA up to 4%) was added basolaterally (250 μl). For basolateral to apical transport (B–A), compounds (10 μM) were added basolaterally and atenolol apically. The Caco-2 plate was then incubated for 2 h at 37 °C in 5% CO_2. Samples were taken from both apical and basolateral compartments and drugs were analysed using a LC–MS/MS system (Quattro Micro spectrometer, Waters) with Turbo ionspray source.

2.4. Bi-directional permeability assay and Pgp inhibition assays

Three Pgp substrates with different lipophilic and protein binding properties were chosen. Digoxin has low lipophilicity properties ($c\log P$ of 0.85) and medium protein binding (between 20 and 30%) (Lisalo, 1977). Verapamil and imipramine have high protein binding around 90% with relatively high lipophilicity ($c\log P$ = 3.9 and 4.8, respectively) (Chung et al., 2001; Walgren and Walle, 1999; Kristensen, 1983). Digoxin, verapamil and imipramine permeability assay was performed at 10 μM in both directions (A–B and B–A). For Pgp inhibition assay, digoxin or imipramine were incubated with and without verapamil (100 μM), added to both compartments.

2.5. LC–MS/MS analysis

A binary high-pressure mixing HP1100 pump (Agilent) was used to deliver a gradient flow to perform the separation on the analytical column. A flow rate of 1 ml/min was used for this column (Atlantis®, Waters Corp.) with dimensions of 2.1 mm × 30 mm, 3 μm particles size. Acetate buffer and acetonitrile both with 0.01% formic acid were used as mobile phases (solvents A and B, respectively). A CTC HTS PAL autosampler (CTC Analytics) was used to inject the samples. A Quattromicro LC Triple-quadrupole mass spectrometer (Waters Corp.) with a Z-spray ionisation source was used. It was operated in the multiple-reaction-monitoring mode. The positive and negative electrospray ionisation modes were both used

depending on the analysed product. In each experiment, three transitions were followed: one for the tested compound, one for atenolol and one for the internal standard. The delivered solvent was a mixture of acetonitrile with water. The calibration consisted in two injections of the calibration sample before and after injection of the four samples with the tested compound. A linear through zero regression, using the area ratio of the compound and the internal standard, was determined and used to back-calculate the concentration of the unknown samples.

3. Results

3.1. Adsorption analysis in classic permeability assay for lipophilic compounds

Seventeen high lipophilic compounds with high protein binding and different *c* log *P* from Laboratories FOURNIER and one reference compound with high permeability, antipyrine, were analysed in a permeability assay in classic conditions (HBSS) with or without cells. Recovery was measured after 2 h of incubation at 37 °C (Table 1). Thirteen lipophilic compounds out of seventeen had recoveries less than or equal to 20% in both conditions, with or without cells and only antipyrine had a recovery of more than 80% after 2 h of incubation. The mean recovery of these seventeen high lipophilic compounds was 20%. Linear regression between recovery of the same compounds after 2 h of incubation at 37 °C in transwell plates without cells and *c* log *P* (Fig. 1) gives a regression value of 0.71.

Table 1
Recovery of 18 laboratoires Fournier compounds after 2 h of incubation in permeability assay conditions with or without cells on transwell plates (grey cells: recovery between 20 and 50% and dark grey cells: recovery lower to 20%)

Compounds	recovery without cells (%)	recovery with cells (%)	cLogP
Compound 1	47 ± 4	60 ± 6	2.59
Compound 2	18 ± 8	15 ± 4	4.3
Compound 3	11 ± 2	13 ± 1	3.97
Compound 4	3.5 ± 2.2	6.5 ± 1.8	4.47
Compound 5	2.1 ± 0.2	4.4 ± 0.2	5.02
Compound 6	0.0 ± 0.0	0.0 ± 0.0	7.14
Compound 7	0.0 ± 0.0	0.0 ± 0.0	7.71
Compound 8	18 ± 6	0.2 ± 0.1	5.93
Compound 9	15 ± 9	13 ± 2	4.21
Compound 10	44 ± 2	57 ± 4	2.78
Compound 11	64 ± 0	51 ± 2	2.77
Compound 12	74 ± 8	32 ± 2	1.08
Compound 13	17 ± 1	21 ± 8	4.12
Compound 14	8.9 ± 2.4	10 ± 2	4.12
Compound 15	14 ± 4	17 ± 5	4.45
Compound 16	22 ± 5	19 ± 5	2.85
Compound 17	21 ± 4	19 ± 1	2.87
antipyrine	88 ± 0	90 ± 4	0.27
mean of 17 compounds	22	20	

LogP was calculated by ACD for all compounds. Each data point represents the mean of two replicates.

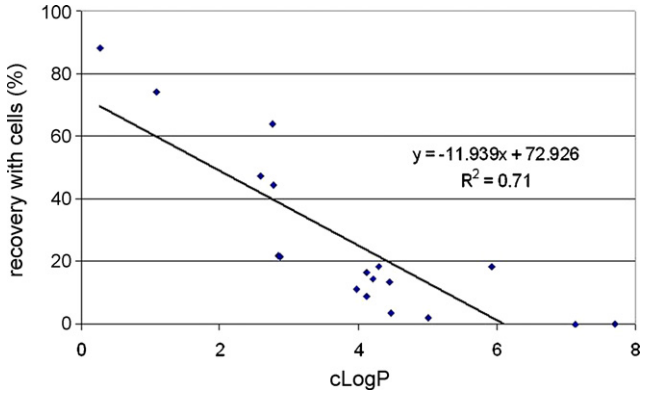

Fig. 1. Correlation between recovery of 18 compounds and LogP calculated by ACD software after 2 h of incubation at 37 °C in transwell plate without cells.

3.2. Permeability assay in different conditions

A set of six highly lipophilic compounds with high protein binding from Laboratories FOURNIER and two references (low permeability compound, ranitidine and high permeability compound, antipyrine) were analysed in permeability assays using different conditions: HBSS buffer or FaSSIF in apical compartment and HBSS with or without BSA in basolateral compartment. Apparent permeability and recovery were measured after 2 h of incubation (Table 2). All assays with HBSS in the apical compartment with or without BSA showed low recovery (<20%) for four out of six highly lipophilic compounds, 38–60% for the other two lipophilic compounds and 37–66% for both references. The mean recovery was around 30% in all assays with HBSS in apical compartment. On the other hand, no compound presented very low recovery (<20%) in all assays using FaSSIF in the apical compartment and the mean recovery was 61 and 75% with 0 and 1% of BSA in basolateral compartment, respectively. In an assay with 4% of BSA in the basolateral compartment, the mean recovery was 100%. Nevertheless, 2 compounds presented aberrant results with recoveries of 184 and 201%. For apparent permeability results, little or no differences were observed with both references but highly lipophilic drugs presented high differences. Generally, addition of BSA in the basolateral compartment increases the apparent permeability results and replacement of HBSS by FaSSIF in apical compartment decreases the permeability results.

3.3. Comparison of permeability and recovery results of highly lipophilic compounds between two different methods

A set of 10 very highly lipophilic compounds from Laboratoires FOURNIER were analysed in permeability assays using two different conditions: HBSS buffer in both compartments versus FaSSIF in apical compartment with HBSS BSA 1% in basolateral compartment. The latter are the new chosen conditions for highly lipophilic compounds (named FaSSIF method as opposed to classic method). Apparent permeability and recovery were measured after 2 h of incubation (Table 3). Only 2 compounds out of 10 have recoveries superior to 20% in classic method. Nine compounds out of ten have recoveries superior to 50% and the last compound has a recovery of 40%.

3.4. Comparison of reference compounds permeability results between two different methods

A set of 35 compounds, for which absorbed fractions in human were available and ranged from 0 to 100%, were selected and tested with the classic method and the FaSSIF method. In this set, com-

Table 2
Permeability assay of eight compounds (six laboratoires Fournier compounds and two commercially references) with different lipophilicity properties in El different conditions: HBSS or FaSSIF in apical compartment and HBSS with or without BSA 1 or 4% in basolateral compartment with recovery analysis (grey cells: recovery between 20 and 50% and dark grey cells: recovery lower to 20%) (A) and apparent permeability calculated (grey cells: high permeability) (B)

A

	cLogP	Recovery (%) after permeability assay in different conditions					
in apical		HBSS	HBSS	HBSS	FASSIF	FASSIF	FASSIF
in basolateral		HBSS	HBSS + BSA1%	HBSS + BSA 4%	HBSS	HBSS + BSA1%	HBSS + BSA 4%
compound 1	4.47	1.9 ± 0.5	6.7 ± 0.4	7.7 ± 0.3	43 ± 13	76 ± 13	*201 ± 10*
compound 2	3.89	5.9 ± 1.5	6.6 ± 0.0	11 ± 2	93 ± 5	64 ± 4	58 ± 0.5
compound 3	3.17	45 ± 3	41 ± 12	38 ± 0	57 ± 21	128 ± 2	87 ± 6
compound 4	4.3	7.1 ± 5.0	11 ± 4	17 ± 8	32 ± 2	57 ± 2	100 ± 3
compound 5	5.03	3.7 ± 1.3	7.4 ± 1.1	11 ± 3	52 ± 11	84 ± 17	*184 ± 18*
compound 6	0.6	59 ± 6	52 ± 5	48 ± 11	64 ± 2	56 ± 9	41 ± 3
ranitidine	1.23	66 ± 12	52 ± 5	37 ± 1	69 ± 20	56 ± 4	57 ± 20
antipyrine	0.27	61 ± 25	48 ± 0.6	51 ± 5	76 ± 4	78 ± 7	72 ± 18
Mean		31	28	28	61	75	100

B

	cLogP	Apparent permeability (10^{-6} cm/sec) after permeability assay in different conditions											
in apical		HBSS		HBSS		HBSS		FASSIF		FASSIF		FASSIF	
in basolateral		HBSS		HBSS + BSA1%		HBSS + BSA 4%		HBSS		HBSS + BSA1%		HBSS + BSA 4%	
compound 1	4.47	34 ± 2	HIGH	76 ± 4	HIGH	82 ± 2	HIGH	0.8 ± 0.4	LOW	28 ± 5	HIGH	19 ± 2	HIGH
compound 2	3.89	44 ± 3	HIGH	76 ± 3	HIGH	79 ± 3	HIGH	2.0 ± 0.3	LOW	32 ± 3	HIGH	25 ± 1	HIGH
compound 3	3.17	67 ± 1	HIGH	75 ± 2	HIGH	77 ± 1	HIGH	38 ± 2	HIGH	53 ± 2	HIGH	50 ± 6	HIGH
compound 4	4.3	68 ± 5	HIGH	70 ± 2	HIGH	76 ± 0	HIGH	3.4 ± 2.4	HIGH	31 ± 5	HIGH	28 ± 6	HIGH
compound 5	5.03	37 ± 5	HIGH	70 ± 7	HIGH	76 ± 1	HIGH	3.3 ± 0.7	HIGH	28 ± 2	HIGH	21 ± 6	HIGH
compound 6	0.6	2.9 ± 0.3	LOW	3.6 ± 0.2	HIGH	5.1 ± 1.2	HIGH	2.5 ± 0.2	LOW	3.7 ± 0.5	HIGH	3.2 ± 0.7	HIGH
ranitidine	1.23	0.1 ± 0.0	LOW	0.1 ± 0.0	LOW	0.2 ± 0.0	LOW	0.1 ± 0.0	LOW	0.2 ± 0.1	LOW	0.2 ± 0.0	LOW
antipyrine	0.27	50 ± 0	HIGH	49 ± 1	HIGH	48 ± 6	HIGH	42 ± 1	HIGH	39 ± 8	HIGH	40 ± 6	HIGH

Each data point represents the mean of two replicates.

pounds have different absorption and transport characteristics: paracellular transport (i.e. cefmetazole and furosemide), or passive transcellular absorption (i.e. terbutaline and antipyrine), or active transcellular permeability by different absorption or efflux transporters (i.e. methylprednisolone, etoposide, quinidine, doxorubicin, ciprofloxacin, ranitidine, sulfasalazine and sulpiride). Furthermore, these compounds have different degrees of lipophilicity (calculated LogP by ACD ranged from −2.84 to 3.57). All compounds were studied at the concentration of 10 μM using both methods on Caco-2 cells at 37 °C for 2 h on a Caliper LifeSciences Staccato platform. A linear regression through zero was established between apparent permeability results determined by both methods (Fig. 2). This relationship leads to a regression value (r^2) of 0.95. Nevertheless, permeability results were lower in the assay using the FaSSIF method compared to the classic method. Effectively, the maximum permeability result obtained with the

Table 3
Permeability assay of 10 laboratoires Fournier compounds with very high lipophilicity property in HBSS and FaSSIF methods with apparent permeability calculated (NC for not calculated because of low recovery and grey cells for high permeability results) and recovery analysis (grey cells: recovery between 20 and 50% and dark grey cells: recovery lower to 20%)

	cLogP	HBSS			FaSSIF		
		Papp (10^{-6} cm/sec)	classification	Recovery (%)	Papp (10^{-6} cm/sec)	classification	Recovery (%)
compound 1	4.3	48 ± 4	High	28 ± 14	25 ± 5	High	81 ± 17
compound 2	4.35	NC	NC	<20	1.2 ± 0.7	Low	40 ± 4
compound 3	4.47	NC	NC	<20	20 ± 6	High	59 ± 16
compound 4	4.8	26 ± 3	High	43 ± 1	18 ± 3	High	97 ± 9
compound 5	5.02	NC	NC	<20	30 ± 7	High	81 ± 10
compound 6	5.22	NC	NC	<20	34 ± 0	High	54 ± 5
compound 7	7.06	NC	NC	<20	8.6 ± 0.5	High	60 ± 4
compound 8	7.4	NC	NC	<20	3.1 ± 0.7	High	63 ± 3
compound 9	8.37	NC	NC	<20	0.6 ± 0.0	Low	74 ± 2
compound 10	9.93	NC	NC	<20	0.2 ± 0.1	Low	76 ± 1

NC: Not Calculated because of low recovery (<20%)

Each data point represents the mean of two to six replicates. NC, not calculated because of low recovery (<20%).

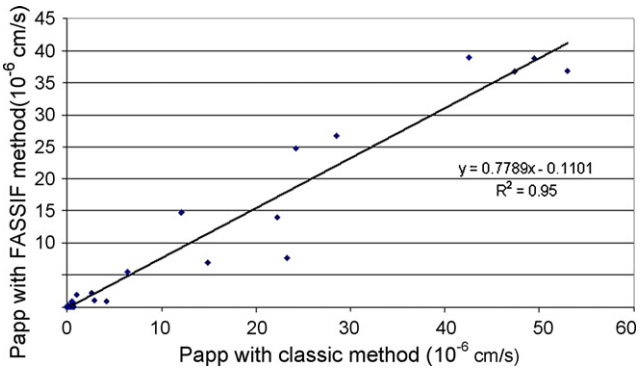

Fig. 2. Correlation of permeability values for 35 compounds in 96-well Caco-2 plates between classic and FaSSIF methods compared.

FaSSIF method was 39×10^{-6} cm/s and 53×10^{-6} cm/s in classic method.

3.5. Correlation with human in vivo absorption data

Correlation between absorbed fraction in human (the literature data: Palm et al., 1998; Kansy et al., 1998; Wessel et al., 1998; Balon et al., 1999; Bircher and Sommer, 1999) and measured permeability values of the 35 compounds was compared between both methods (Table 4, Figs. 3 and 4). The same logarithmic relationship between absorbed fraction in human and measured permeability values of these compounds was established with both methods. Only 3 outliers out of 35 compounds (dicloxacillin, sulfamethizole and norfloxacin) were observed using the classic method and 2 outliers with FaSSIF method (dicloxacillin and sulfamethizole).

3.6. Permeability assay on Pgp substrates

Digoxin, verapamil and imipramine were analysed in both directions on both models. Results showed that all three compounds presented similar results between classic and FaSSIF methods. An efflux ratio was observed for all three compounds of 1.5 and 2, respectively and was not calculable for digoxin since the apparent permeability result from A to B was 0.0×10^{-6} cm/s. Nevertheless, the permeability result in secretory direction was higher than

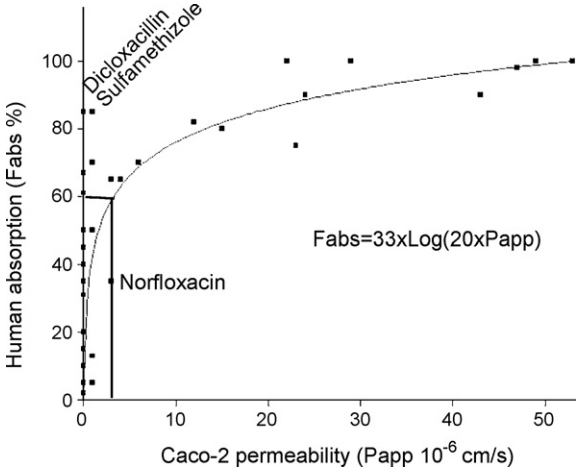

Fig. 3. Correlation between known human absorbed fraction (Fabs) (the literature data) and Caco-2 permeability data in 96-well plates (Papp) for 35 compounds with classic method (HBSS). Permeability studies were conducted on an automation platform at a concentration of 10 μM concentration and incubated at 37 °C for 2 h. Each data point represents the mean of two replicates.

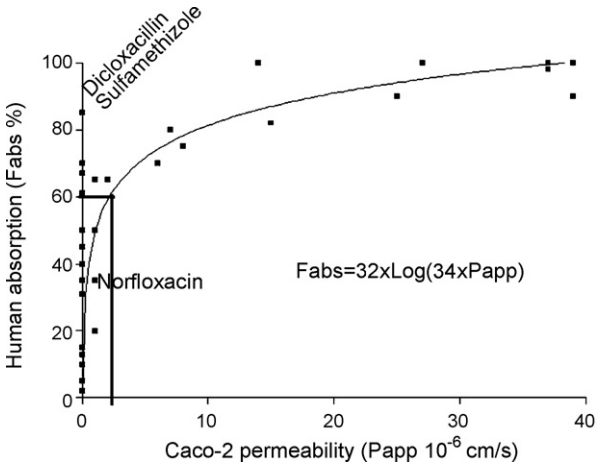

Fig. 4. Correlation between known human absorbed fraction (Fabs) (the literature data) and Caco-2 permeability data in 96-well plates (Papp) for 35 compounds with FaSSIF method (FaSSIF in apical compartment and HBSS + 1% BSA in basolateral compartment). Permeability studies were conducted on an automation platform at a concentration of 10 μM and incubated at 37 °C for 2 h. Each data point represents the mean of two replicates.

that in absorption direction, 4.6×10^{-6} cm/s and 0.0×10^{-6} cm/s, respectively. With addition of verapamil in both compartments, efflux ratio was around 1 for digoxin and imipramine. Furthermore, with the FaSSIF method, the mean compound recovery after 2 h of incubation was 75% for absorption direction and 69% for secretory direction. With the classic method, the mean compound recovery after 2 h of incubation was 45% for both directions (Table 5).

4. Discussion

As mentioned beforehand, the transport of drugs across the intestinal epithelial cell barrier is a major determinant of *in vivo* bioavailability. The model developed by Fogh and Trempe (1975) using the measurement of compound flux across a monolayer of Caco-2 cells has become the most widespread method used to assess intestinal permeability and its reliability for prediction of oral absorption has been proven (Artursson and Karlsson, 1991). Generally speaking, Caco-2 permeability assays permit classification of compounds in two distinct classes, low and high, allowing prediction of drug absorption in the early stages of development (Artursson and Karlsson, 1991; Lau et al., 2004; Matsson et al., 2005). Nowadays, compounds synthesized by chemical groups in pharmaceutical companies are increasingly lipophilic (Lipinski et al., 1997). Caco-2 assays are traditionally performed in HBSS-like buffers, which have highly hydrophilic properties. Lipophilic compounds are poorly or not at all soluble and may be adsorbed on transwell plates in these conditions. Effectively, recovery of lipophilic compounds in a classic permeability assay was on average of 20%. The fact that recovery was very low with or without cells is the consequence of adsorption on transwell plates and/or solubility of compounds rather than metabolism in cells. Furthermore, linear regression between recovery of 18 compounds after 2 h of incubation at 37 °C in transwell plates without cells and $c\log P$ leads to a regression value r^2 of 0.71 confirming a relationship between recovery in Caco-2 assay and lipophilicity of compounds.

Conditions of permeability assays on Caco-2 cells need to be changed to allow analysis of lipophilic compounds. Addition of different solubilizers in the apical compartment has been tested (Takahashi et al., 2002). But, the majority of them negatively affect the viability of Caco-2 monolayers. Although, solubilizers effectively improve solubility of lipophilic compounds in the

Table 4
Permeability values for 35 compounds in 96-well Caco-2 plates using classic or FaSSIF methods compared with known human absorption values (the literature data)

Compound	*c* log *P*	Human absorption (Fabs, %)	Caco-2 permeability Classic method mean Papp ± S.D. (10^{-1} cm/s)	Caco-2 permeability FASSIF method mean Papp ± S.D. (10^{-1} cm/s)
Ceftazidime	−2.84	2	0.0 ± 0.0	0.2 ± 0.1
Cefazolin	1.13	5	0.0 ± 0.0	0.0 ± 0.0
Cefuroxime	0.47	5	0.0 ± 0.0	0.0 ± 0.0
Doxorubicin	3.07	5	0.6 ± 0.6	0.0 ± 0.0
Cefmetazole	0.97	10	0.1 ± 0.0	0.0 ± 0.0
Cefoperazone	1.43	10	0.1 ± 0.1	0.1 ± 0.0
Sulfasalazine	3.18	13	0.7 ± 0.7	0.0 ± 0.0
Cefoxitin	0.63	15	0.0 ± 0.0	0.0 ± 0.0
Methotrexate	−0.24	20	0.5 ± 0.1	0.8 ± 0.1
Sulpiride	0.45	31	0.2 ± 0.0	0.1 ± 0.0
Nadolol	1.29	35	0.3 ± 0.1	0.1 ± 0.0
Norfloxacin	1.48	35	2.9 ± 1.2	1.1 ± 0.0
Enalapril	2.43	40	0.1 ± 0.0	0.1 ± 0.0
Ampicillin	1.35	45	0.2 ± 0.2	0.3 ± 0.0
Amiloride	1.08	50	0.7 ± 0.2	0.5 ± 0.0
Etoposide	0.30	50	0.0 ± 0.0	0.0 ± 0.0
Ranitidine	1.23	50	0.3 ± 0.1	0.3 ± 0.0
Furosemide	3.00	61	0.5 ± 0.4	0.0 ± 0.0
Ciprofloxacin	1.31	65	2.6 ± 0.7	2.2 ± 0.1
Tetracycline	−1.47	65	4.2 ± 1.5	0.9 ± 0.5
Captopril	0.27	67	0.0 ± 0.0	0.0 ± 0.0
Enoxacin	1.21	70	6.4 ± 1.4	5.5 ± 0.9
Terbutaline	0.48	70	0.7 ± 0.7	0.2 ± 0.0
Guanabenz	2.57	75	23 ± 3	7.7 ± 2.4
Quinidine	3.44	80	15 ± 1	7.0 ± 0.4
Methylprednisolone	1.99	82	12 ± 4	15 ± 2
Dicloxacillin	3.02	85	0.2 ± 0.1	0.0 ± 0.0
Sulfamethizole	0.51	85	1.0 ± 0.2	1.9 ± 0.3
Paracetamol	0.34	90	24 ± 2	25 ± 3
Phenytion	2.52	90	43 ± 2	39 ± 1
Antipyrine	0.27	98	47 ± 2	37 ± 2
Bnompluéniramine	3.57	100	22 ± 2	14 ± 2
Caffeine	−0.13	100	50 ± 2	39 ± 4
Testosterone	3.48	100	53 ± 3	37 ± 2
Theophylline	−0.18	100	29 ± 5	27 ± 0

Each data point represents the mean of two replicates.

apical compartment, but they have no effect on adsorption in both compartments. Ingels et al. (2002, 2004) first demonstrated that replacement of HBSS by simulated intestinal fluid in apical compartment improves both solubility and reduces adsorption of lipophilic compounds. Furthermore, different authors have shown that addition of up to 4% BSA in the basolateral compartment during the permeability assay improves recovery and the apparent permeability results (Aungst et al., 2000; Yamashita et al., 2000; Krishna et al., 2001). For highly lipophilic compounds with high protein binding properties, a combination of both conditions might be the best solution to improve permeability assay and to better reflect *in vivo* absorption. The major problem of lipophilic drugs is the adsorption phenomenon in both compartments, which can be reduced in both by using simulated intestinal fluid in the apical compartment and addition of BSA in the basolateral compartment. To test this hypothesis, different conditions in the apical and basolateral compartments were tested in a permeability assay of six lipophilic drugs from laboratoires Fournier and two reference molecules, ranitidine and antipyrine. The mean recovery was around 30% in all assays with HBSS in apical compartment. On the other hand,

Table 5
Permeability assay on Pgp substrates with or without Pgp inhibitor in both directions on classic (A) and FaSSIF (B) methods

Pgp substrates	Pgp inhibitor	Papp A to B (10^{-6} cm/s)	Recovery (%)	Papp B–A (10^{-6} cm/s)	Recovery (%)	Efflux ratio
A						
Digoxin	–	0 ± 0	37	6.3 ± 0.4	37	NC
	Verapamil	3.2 ± 0.8	68	3.6 ± 0.4	65	1.1
Imipramine	–	26 ± 1	43	45 ± 9	46	1.7
	Verapamil	24 ± 1	83	25 ± 1	93	1.0
Verapanil	–	20 ± 0	43	43 ± 2	42	2.2
B						
Digoxin	–	0 ± 0	91	5.0 ± 0.1	86	NC
	Verapamil	4.6 ± 6.4	97	5.1 ± 0.2	58	1.1
Imipramine	–	19 ± 1	90	39 ± 9	73	2.0
	Verapamil	24 ± 1	77	26 ± 1	98	1.1
Verapamil	–	23 ± 2	88	34 ± 2	66	1.5

Each data point represents the mean of two replicates. NC, not calculated.

with FaSSIF in the apical compartment, the mean recovery was 61 and 75% with 0 and 1% of BSA in basolateral compartment, respectively. Increasing the quantity of BSA to 4% (w/v) in the basolateral compartment causes problems during the LC–MS/MS analysis as proteins can interfere with the analytical method. Recovery after 2 h of incubation was increased by using FaSSIF in the apical compartment and was improved when BSA was added in the basolateral compartment. Furthermore, as a general rule for the eight tested compounds, addition of BSA in the basolateral compartment increased the apparent permeability results. However, when HBSS was replaced by FaSSIF in the apical compartment, without changing the conditions in the basolateral compartment, the permeability values decreased as a result of adsorption. Effectively, addition of BSA in only basolateral compartment decreased adsorption in this compartment but not in the apical compartment, therefore resulting in an apparent increase of permeability. On the other hand, keeping HBSS in the basolateral compartment and replacing it by FaSSIF in the apical compartment decreased adsorption apically but not basolaterally, resulting in a decrease of apparent permeability. Consequently, replacement of HBSS by FaSSIF in apical compartment, as proposed by Ingels et al. (2002, 2004), only improved adsorption in apical compartment and solubility of high lipophilic compounds but distorts the permeability results by decreasing them due to adsorption in basolateral compartment. In the same way, BSA added only in basolateral compartment, as proposed by Aungst et al. (2000), Yamashita et al. (2000) or Krishna et al. (2001) increased apparent permeability results as a result of adsorption in apical compartment. Therefore, the best model for high lipophilic compounds seems to entail the use of FaSSIF in the apical compartment and addition of BSA 1% in the basolateral compartment, which decreases adsorption in both compartments. To improve this method for high lipophilic compounds, 10 compounds from laboratoires Fournier with very high lipophilic properties ($c\log P$ between 4 and 10) were performed with both methods, classic and FaSSIF. With classic method, only two compounds out of 10 had permeability results because only both these compounds had recoveries higher than 20% (28 and 43%). With FaSSIF method, all compounds had recoveries higher than 20% and the lowest recovery was 40%. FaSSIF method improves permeability results and especially recovery for highly lipophilic compounds.

Nevertheless, to check the relevance of the permeability results in these new conditions, 35 compounds, with different permeability and lipophilicity properties, were analysed in both conditions (classic method versus FaSSIF in apical compartment with BSA 1% in basolateral compartment). Linear regression through zero between apparent permeability results determined by both methods leads to a regression value (r^2) of 0.95, confirming a good correlation between both methods. Nevertheless, all permeability results in assays using the FaSSIF method were lower compared to classic method. A hypothesis of these results is that bile salts present in FaSSIF in apical compartment should increase osmolarity of apical buffer, increase osmotic pressure between both compartments and thus decrease the passive transport of compounds. A classic permeability assay on Caco-2 cells allows ranking compounds in two classes: low permeability with Papp $< 3 \times 10^{-6}$ cm/s, corresponding to an absorbed fraction in human of less than 60%, and high permeability with Papp $\geq 3 \times 10^{-6}$ cm/s, representing an absorbed fraction in human of more than 60%. These results on classification were in accordance with those obtained by Artursson and Karlsson (1991), Lau et al. (2004) and Matsson et al. (2005). The lower permeability results obtained with the FaSSIF method could have implied altering the limit between "low" and "high" permeability classes, as determined by a classic permeability assay. However, the same logarithmic relationship between absorbed fraction in human and measured permeability values of these compounds was established for both methods. The fact that permeability results were lower with the FaSSIF method compared to a classic method did not however shift this limit between low and high permeability classes with a FaSSIF method. This limit could remain at 3×10^{-6} cm/s. Only 3 outliers out of 35 compounds (dicloxacillin, sulfamethizole and norfloxacin) were observed with the classic method and 2 outliers with the FaSSIF method (dicloxacillin and sulfamethizole). These results confirm that the new conditions could be used in order to have results as relevant as those observed with a classic method.

Nevertheless, taurocholate present in FaSSIF buffer is known to inhibit Pgp transport as described by authors (Ingels et al., 2002, 2004). Digoxin, imipramine and verapamil were used to check the functionality of Pgp on Caco-2 cells in both directions using classic and FaSSIF methods. These three compounds have different protein binding and lipophilicity properties: digoxin is less lipophilic with lower protein binding properties than imipramine and verapamil. Permeability results are similar between both methods. Furthermore, efflux ratio was observed to the same extent with both methods. In this case, taurocholate present in FaSSIF had no inhibitory effect on efflux transport in this model. Furthermore, compound recovery after 2 h of incubation is better with the FaSSIF method than with a classic method for these three compounds. The Pgp inhibition by taurocholate observed by Ingels et al. (2002, 2004) with only HBSS replacement by FaSSIF in apical compartment could reflect distorbed apparent permeability results due to adsorption in basolateral compartment. Thus, the FaSSIF method is a very good alternative for the permeability analysis of lipophilic compounds.

In conclusion, Caco-2 cell permeability assay with FaSSIF in apical compartment and HBSS with BSA 1% in basolateral compartment has proved to be a reliable method for all compounds, including even highly lipophilic compounds with high protein binding. Effectively during permeability assays, this method reduces adsorption in both compartments, improving recovery and consequently, apparent permeability results after 2 h of incubation. Furthermore, this method allows analysing active transport since all tested compounds with active transport gave the same permeability results with both methods and the permeability results of these compounds were correlated with absorbed fraction in human. It is, nevertheless, imperative that batches of Caco-2 cells used in different laboratories are well characterised as regards transporters.

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