Caco-2 cells and Biopharmaceutics Classification System (BCS) for prediction of transepithelial transport of xenobiotics (model drug: caffeine)

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Abstract

OBJECTIVES: The Caco-2 cell monolayer model is widely used as a standard screening tool for studying the mechanisms of cellular drug transport. Caffeine was chosen as a model drug and is supposed to be class I of the Biopharmaceutics Classification System (BCS). Our study was conducted 1) to characterize the mechanisms of caffeine transport across the intestinal barrier, 2) to classify caffeine according to BCS, 3) to predict drugs intestinal absorption in humans.

METHODS: Caffeine transport (0.1, 0.3, 1 and 10 mmol/l) was studied in Caco-2 cell monolayer in apical to basolateral (AP-BL) and basolateral to apical (BL-AP) direction, under iso-pH 7.4 and pH-gradient (6/7.4) conditions. The relative contribution of the paracellular route was estimated using Ca^{2+}- free transport medium (opening tight junctions).

RESULTS: The caffeine transport was linear with time, transport direction and pH independent, displaying non-saturable (first-order) kinetics, with high permeability coefficient (P_{app}): in AP-BL direction P_{app} = 46.3–53.5 × 10^{-6} cm/s; in BL-AP direction P_{app} = 45.6–49.4 × 10^{-6} cm/s. Thus, the transport seems to be transcellular mediated by passive diffusion. Using Ca^{2+}- free transport medium tight junctions were opened (confirmed by increased P_{app} of mannitol) but the caffeine P_{app} was not changed. Thus, the paracellular route is only a minor way of caffeine transport.

CONCLUSION: High solubility and high permeability of caffeine rank it among class I of BCS and well absorbed compounds.

ORIGINAL ARTICLE

Abbreviations & units

AP-BL	apical-to-basolateral
BL-AP	basolateral-to-apical
BCS	Biopharmaceutics Classification System
DMEM	Dulbecco´s modified Eagle´s medium
ECACC	European Collection of Cell Cultures
Fa	fraction absorbed
FDA	US Food and Drug Administration
HBSS	Hanks´ balanced salt solution
HPLC	High performance liquid chromatography
J	flux rate (nmol/min/cm^2)
P_{app}	apparent permeability coefficient (cm/s)

INTRODUCTION

Caco-2 cell monolayers have been widely accepted as a potent *in vitro* model membrane for the rapid screening of the intestinal drug absorption (Hidalgo *et al.* 1989). The transport of drugs across the intestinal epithelium may occur by one or more of four different routes: the passive transcellular and paracellular route, the carrier mediated route and by transcytosis. In general, passive diffusion is the main mechanism for absorption of many lipophilic compounds, while the carrier-mediated process governs the absorption of transporter substrates.

Amidon *et al.* (1995) recognized the fundamental parameters controlling the rate and extent of oral drug absorption and devised Biopharmaceutics Classification System (BCS) that categorized drugs into four classes according to their solubility and permeability. The objective of the BCS is to predict *in vivo* pharmacokinetic performance of drug products from *in vitro* measurements of permeability and solubility (Amidon *et al.* 1995). The BCS has provided a simple and facile scientific framework for drug development and may help decisions to obtain regulatory waivers for bioequivalence studies.

This methodological pre-work is the part of the research targeted to experimental mapping of the bacterial probiotics influence on absorptive and exsorptive barrier mechanisms in the intestine (Kvetina *et al.* 2008). The purpose of this pre-work is to use the BCS for choice of acceptable drug models that would represent particular transport types. As a model drug caffeine (1,3,7-trimethylxanthine) was chosen.

The objectives of this pre-work were: 1) to determine whether the caffeine transport across the Caco-2 monolayer involves an active or a passive mechanism, 2) to evaluate the transcellular and paracellular pathway involved in caffeine transport, 3) to determine effects of pH on the caffeine transport, 4) to classify caffeine according to BCS, and 5) to contribute to prediction of xenobiotics intestinal absorption in humans.

MATERIAL AND METHODS

Materials. For culturing cells material was purchased from PAA Laboratories (BioTech, Praha, Czech Republic) or Gibco Invitrogen (KRD, Praha, Czech Republic). ^{14}C-mannitol (100 μCi/ml) from Moravek Radiochemical and Biochemicals (MGP Zlín, Czech Republic) and all other chemicals were obtained from Sigma-Aldrich (Praha, Czech Republic).

Caco-2 cells. Caco-2 cells (ECACC) were cultured in a standard manner (Bourdet & Thakker, 2006) in plastic tissue culture flasks in DMEM at 37 °C in an atmosphere of 5% CO_2 and 90% relative humidity. For transport studies, Caco-2 cells (passages 75–78) were seeded onto the Transwell inserts at a density of 2.5×10^5 cells/cm^2 and grown to late confluence (21-24 days).

Cell viability assay. Cell viability was determined by trypan blue exclusion method (Nadova *et al.* 2008). Briefly, 24 h after seeding Caco-2 cells onto Petri dishes (2×10^5 cells/cm^2), caffeine 10 mmol/l or transport medium (control) was added and incubated for next 2 h. Then the cells were released and incubated with 0.4% Trypan blue for 5 min and counted by using microscopy.

Monolayer integrity assessment. Before the start of the experiment, the monolayer integrity was checked by 500 μmol/l phenol red permeability/1 h (Fleet & Wood, 1999). During the transport studies, the integrity was confirmed simultaneously by ^{14}C-mannitol (0.5 μCi/ml) permeability (Artursson *et al.* 1996).

Transport studies. Transport experiments were performed as previously described (Walle & Walle, 1998). Briefly, the inserts were rinsed twice and equilibrated with prewarm HBSS at 37°C for 30 min before the transport studies. The transport AP-BL experiments were initiated by replacing the transport medium with the diluted caffeine (0.1, 0.3, 1 and 10 mmol/l) in the transport medium on the apical side. The samples from the basolateral compartment were withdrawn at 30, 60, 90 and 120 min for HPLC analysis. Similarly, for BL-AP transport, the drug was added to the basolateral compartment and samples from the apical compartment withdrawn in the same time intervals. To determine paracellular component of the caffeine (0.3 mmol/l) transport, the HBSS Ca^{2+}- free medium was used. Influence of pH gradient (pH apical 6/ basolateral 7.4) was studied only in the concentration 0.3 mmol/l of caffeine.

Apparent permeability coefficient P_{app} (cm/s) was calculated (Artursson & Karlsson, 1991):

$$\mathbf{P_{app} = (dQ/dt) \times (1/A \times C_0),}$$

where dQ/dt is the permeability rate, the amount of drug appearing in the receiver compartment in function of time (nmol/s), C_0 is the initial concentration in the donor chamber (nmol/ml), and A is the surface area of the monolayer (cm^2). Transport in both directions across monolayer enables to calculate (Ungell & Karlsson, 2004)

$$\mathbf{P_{app}\ uptake\ ratio = P_{app}\ AP\text{-}BL/\ P_{app}\ BL\text{-}AP}.$$

HPLC analysis of caffeine. The caffeine quantification was performed by a method of Pastera *et al.* (1999) with slight modifications. The HPLC with a gradient elution and UV detection at 275 nm was used.

Table 1. Permeability, percent transport and P_{app} uptake ratio of caffeine across Caco-2 monolayer.

Treatment		P_{app} ($\times 10^{-6}$ cm/s)		Transport (%)		Uptake ratio
		AP - BL pH 7.4 - 7.4	BL - AP pH 7.4 - 7.4	AP - BL pH 7.4 - 7.4	BL - AP pH 7.4 - 7.4	
Phenol red		1.3 ± 0.7		0.4 ± 0.2		
Mannitol		1.5 ± 0.1		1.1 ± 0.1		
Mannitol + caffeine	0.3 mmol/l	1.5 ± 0.3		1.1 ± 0.2		
Mannitol + caffeine	10 mmol/l	1.3 ± 0.1		0.9 ± 0.1		
Caffeine	0.1 mmol/l	46.3 ± 2.0	45.6 ± 3.1	33.3 ± 1.4	25.3 ± 1.7^{+}	1.02
Caffeine	0.3 mmol/l	46.3 ± 1.0	46.9 ± 3.0	33.3 ± 0.7	26.0 ± 1.6^{+}	1.01
Caffeine	1 mmol/l	53.0 ± 1.4	49.4 ± 2.7	38.2 ± 1.0 ^{*1,2}	27.3 ± 1.5^{+}	0.93
Caffeine	10 mmol/l	53.5 ± 1.9	46.3 ± 1.3	38.5 ± 1.3 ^{*1,2}	25.6 ± 0.7^{+}	1.16
		AP - BL pH 6 - 7.4		AP - BL pH 6 - 7.4		
Caffeine	0.3 mmol/l	44.7 ± 3.7		32.2 ± 2.7		

Values are represented as mean ± SD. Effect of caffeine concentration on P_{app} values and % transport –*significant difference: ^{1}versus 0.1 mmol/l, ^{2}versus 0.3 mmol/l ($p<0.05$). $^{+}$AP-BL versus BL-AP caffeine P_{app} and percent transport ($p<0.001$). Effect of pH conditions on caffeine P_{app} and % transport (0.3 mmol/l): $^{\#}$significant difference: iso-pH versus pH gradient (pH 6/7.4) condition ($p<0.05$). Uptake ratio= P_{app} BL-AP/P_{app} AP-BL.

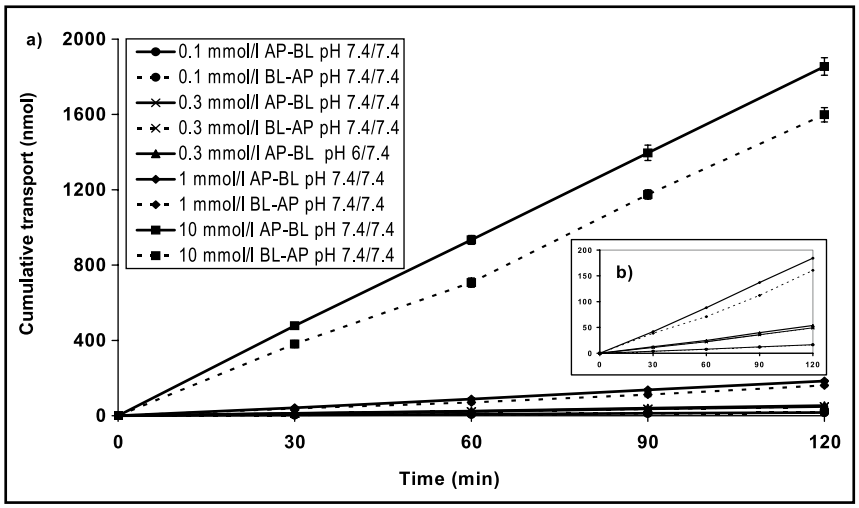

Figure 1:
a) The cumulative transport (apical to basolateral direction AP-BL – solid line and basolateral to apical BL-AP – dashed line) of caffeine (0.1, 0.3, 1 and 10 mmol/l) under iso-pH (pH AP/BL: 7.4/7.4) and pH-gradient (pH AP/BL: 6/7.4) conditions.
b) The cumulative transport of caffeine (0.1, 0.3, 1 mmol/l) in detail. Each point represents the mean ± SD.

Statistical analysis. All treatments were carried out at least in triplicate. All values are represented as mean ± standard deviation (SD). One-way or two-way analysis of variance (ANOVA) with Sheffé´s post hoc test or Student's *t*-test was used. Differences were considered significant at $p<0.05$.

RESULTS

Cell viability assay. Caffeine 10 mmol/l did not influence Caco-2 cell viability in comparison with viability of Caco-2 cells incubated with transport medium (98.0 ± 0.3% vs. 98.6 ± 0.4%, not significant).

Monolayer integrity assessment. The monolayer integrity (***Table 1***) was appropriate (mannitol P_{app} = 1.48×10^{-6} cm/s). There was no effect of caffeine on the P_{app} of mannitol.

Transport studies. The caffeine cumulative transport was linear for up to 2 h over the concentration range examined and was pH-independent (***Fig. 1***). The percent amount of transported caffeine reached around 30% (***Table 1***). The percent transport in BL-AP direction (***Table 1***) was significantly lower then in AP-BL direction ($p<0.001$). There were also seen significant differences in % AP-BL transport in concentration 1 and 10 mmol/l versus 0.1 mmol/l and 0.3 mmol/l ($p<0.05$, but not $p<0.001$). Under the pH-gradient the transported amount of caffeine was not changed (***Table 1***). P_{app} of caffeine was independent of concentration over the entire concentration range (0.1–10 mmol/l) and was 46.3–53.5 $\times 10^{-6}$ cm/s in AP-BL direction and 45.6–49.4 $\times 10^{-6}$ cm/s in BL-AP direction for all used concentrations (***Table 1***). Furthermore, the calculated P_{app} uptake ratios oscillated around 1 (***Table 1***). The

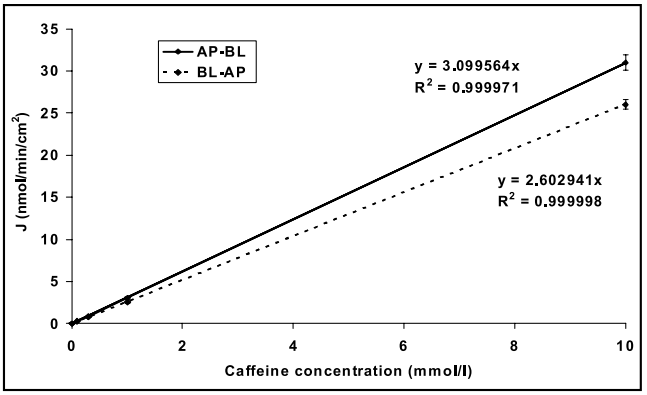

Figure 2. Flux rate vs. caffeine concentration. AP-BL, apical to basolateral flux; BL-AP, basolateral to apical flux. Each point is the mean ± SD.

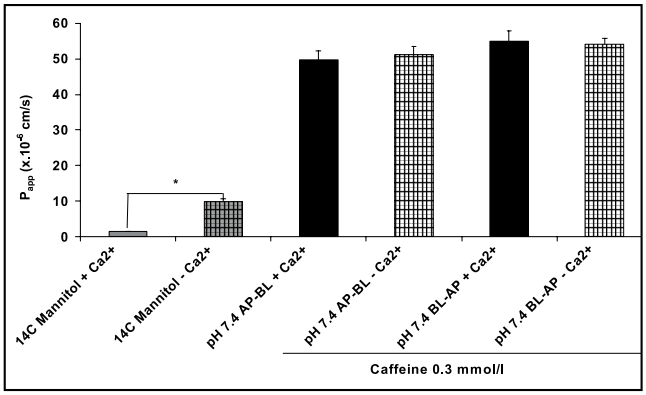

Figure 3. Caffeine and ^{14}C-mannitol apical-basolateral (AP-BL) permeability in presence (+ Ca^{2+}) or absence (- Ca^{2+}) in transport medium. Each column represents the mean ± SD of permeability coefficient (P_{app}). *Statistical significance ($p<0.05$).

AP-BL and BL-AP flux rates were a simple linear function of caffeine concentrations indicating passive diffusion (***Fig. 2***). The caffeine recovery (Ungell & Karlsson, 2004) reached 94–107% for both directions.

The transport of the ^{14}C-mannitol (paracellular marker) was significantly enhanced (6.75 times) by the use of the HBSS Ca^{2+}- free transport medium in comparison with using transport medium with Ca^{2+}. However, the caffeine P_{app} (0.3 mmol/l) was not changed in both transport directions (***Fig. 3***) and oscillated around 50×10^{-6} cm/s.

DISCUSSION

The Caco-2 cell monolayer as a model of intestinal absorption was utilized in this study. In cell culture, Caco-2 cells grow on microporous membranes and spontaneously differentiate to form intact monolayer. The drug permeability across Caco-2 monolayer is expected to correlate well with that of intestinal membrane *in vivo* (Artursson & Karlsson, 1991). The Caco-2 cells are grown to reach the confluence with well-developed tight junctions (21 days). The presence of well-developed tight junction and a tight epithelium can be checked by the permeability of hydrophilic marker molecules (e.g., mannitol) as hydrophilic molecules are not distributed to the cell membrane to a large extent, their transport across the epithelial monolayer is limited to the paracellular route (Artursson *et al.* 1996). In our earlier studies we reported also phenol red as a reliable tool for studying monolayer integrity (Smetanova *et al.* 2008). In our present work transepithelial transport of mannitol and phenol red showed adequate integrity of the monolayer. Caffeine was not toxic to the Caco-2 cells as caffeine did not influence viability of cells and transport of mannitol.

This methodological pre-work is the part of the research targeted to experimental mapping of the bacterial probiotics influence on absorptive and exsorptive barrier mechanisms in the intestine. In our experiments caffeine as a model drug was used in a wide range of concentrations: 0.1–10 mmo/l and caffeine transport was monitored for a period of 120 min. During the experiments the recovery of caffeine was > 90% in the overall experiments confirming that there was no degradation, metabolism or binding to the cells.

The first objective was to determine whether the caffeine transport across the intestinal epithelium involved an active or passive mechanism. The data demonstrated that cumulative caffeine transport (over the concentration range examined) in Caco-2 cell monolayers was linear with time without any saturation, dependent on concentration and independent of transport direction. Caffeine transport in the absorptive (AP-BL) and the secretory (BL-AP) direction was not significantly different as showed by similar P_{app} in both directions and by P_{app} uptake ratios around 1 for all concentrations used. Also the AP-BL and BL-AP flux rates (J) with linear course and no difference between both directions indicated passive diffusion. There seems to be a slight preference in the percent transport for apical to basolateral flux (slightly lower percent transport from basolateral compartment than from apical compartment), which may be explained by the differences in the surface areas of the apical and basolateral side of the monolayer and volume differences of the donor compartments. The differences seen in % transport in concentration 1 and 10 mmol/l versus 0.1 mmol/l and 0.3 mmol/l ($p<0.05$, but not $p<0.001$) could have been caused by the fact that 0.1 mmol/l and 0.3 mmol/l were not done on the same desk as the concentrations 1 and 10 mmol/l. High permeability coefficients in both directions P_{app} (AP-BL) = $46.3–53.5 \times 10^{-6}$ cm/s and P_{app} (BL-AP) = $45.6–49.4 \times 10^{-6}$ cm/s indicated passive transcellular transport. To further investigate the caffeine absorption mechanism, the effect of pH was studied. Different pH in apical compartment (pH gradient) did not influence the transport caffeine in comparison with iso-pH conditions.

The next objective of this work was to evaluate whether this passive transepithelial transport of caffeine occurred via the paracellular or transcellular route. Ca^{2+}-free medium was used to distinguish these two ways of transport (McMillan *et al.* 2005). The mannitol transport as a paracellular transport marker was enhanced (6.5 times) in this conditions but transport of caffeine remained unchanged. That is clear evidence for paracelullar transport not playing important role in mechanism of caffeine transport. Thus, the paracellular route is only a minor way of caffeine transport.

BCS divided drug compounds into four classes based on their solubility and permeability.

As for solubility caffeine with solubility 21.7 mg/ml water (data from literature – Merck index 2001) is soluble according to semiquantitative range for solubility (Lipinski, 2004).

According to our reported caffeine P_{app} values around 50×10^{-6} cm/s, caffeine can be determined as a highly permeable drug and this high P_{app} value ranks caffeine among well absorbed compounds (Yee, 1997). This statement is in a good agreement with publicated data reporting that after oral administration caffeine is rapidly and completely absorbed from the gastrointestinal tract, and c_{max} is reached at about 1 h (Carrillo & Benitez, 2000), and bioavailability is 100% in humans (Yee, 1997). The good correlation between permeability across Caco-2 cell monolayer, solubility and oral bioavailability in humans has been demonstrated (Ungell & Karlsson, 2004).

Caffeine as highly soluble and highly permeable drug can be categorized into the class I of BCS. The results have shown Caco-2 cells as an appropriate model that enables to study permeability. Drug categorization into BCS system enables to predict drug transepithelial transport.

CONCLUSION

The results showed that caffeine transport is linear with time, independent of transport direction and of pH, displaying non-saturable (first-order) kinetics, with high permeability coefficient. The transport seems to be transcellular mediated by passive diffusion. Using Ca^{2+}- free transport medium, the caffeine P_{app} was not changed. The paracellular route is only a minor way of caffeine transport. Estimated high solubility and high permeability of caffeine rank it into class I of BCS which is in a good agreement with the literature data. It can be supposed that the oral dose fraction of caffeine absorbed in humans is high. The results showed that Caco-2 monolayer model and BCS are suitable tools for prediction of drug transepithelial transport.

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REFERENCES

1 Amidon GJ, Lennernäs H, Shap VP, Crison JR (1995). A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability. Pharm Res. **12**: 413–420.
2 Artursson P, Karlsson J (1991). Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. Biochem Biophys Res Comm. **175**: 880–885.
3 Artursson P, Karlsson J, Ocklind G, Schipper N (1996). Studying transport process in absorptive epithelia. In: Shaw AJ, editor. Epithelial cell culture - a practical approach. Oxford University Press. p. 111–133.
4 Bourdet DL, Thakker DR (2006). Saturable absorptive transport of the hydrophilic organic cation ranitidine in Caco-2 cells: role of pH-dependent organic cation uptake system and P-glycoprotein. Pharm Res. **23**: 1165–1177.
5 Carrillo JA, Benitez J (2000). Clinically significant pharmacokinetic interactions between dietary caffeine and medications. Clin Pharmacokinet. **39**: 127–153.
6 Fleet JC, Wood RJ (1999). Specific $1,25(OH)_2D_3$-mediated regulation of transcellular calcium transport in Caco-2 cells. Am J Physiol. **276**: G958–G964.
7 Hidalgo IJ, Raub TJ, Borchardt RT (1989). Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology. **96**: 736–749.
8 Kvetina J, Kunes M, Bures J, Kopacova M, Tacheci I, Spelda S, et al (2008). The use of wireless capsule enteroscopy in a preclinical study: a novel diagnostic tool for indomethacin-induced gastrointestinal injury in experimental pigs. Neuroendocrinol Lett. **29**: 763–769.
9 Lipinski Ch (2004). Aqueous solubility in discovery, chemistry, and assay changes. In: van de Waterbeemd, Lennernäs H, Artursson P, editors. Drug bioavailability. Weinheim: WILEY-VCH. p. 215–231.
10 Merck Index (2001), Caffeine. In: O´Neil MJ, Smith A, Heckelman PE, editors. Merck Index. An encyclopedia of chemicals, drugs, and biologicals, 13th ed., New Jersey: Merck & Co., Inc. p. 1639.
11 McMillan JM, Walle UK, Walle T (2005). S-adenosyl-L-methionine: transcellular transport and uptake by Caco-2 cells and hepatocytes. J Pharm Pharmacol. **57:** 599–605.
12 Nadova S, Miadokova E, Alfoldiova L, Kopaskova M, Hasplova K, Hudecova A, et al (2008). Potential antioxidant activity, cytotoxic and apoptosis-inducing effects of Chelidonium majus L. extract on leukemia cells. Neuroendocrinol Lett. **29**: 649–652.
13 Pastera J, Anzenbacher P, Fiala Z (1999). Phenotyping of cytochrome P-450 1A2 and N-acetyltransferase (NAT2) using the in vivo caffeine test as a tool for determining individual susceptibility to selected xenobiotics. Acta Medica (Hradec Kralove). **42** (Suppl.): 3–5.
14 Smetanova L, Stetinova V, Kvetina J, Svoboda Z (2008). The use of ^{14}C- mannitol and phenol red in the assessment of the Caco-2 monolayer integrity. Prague Medical Report. **109** (Suppl.): S109–111.
15 Ungell A-L, Karlsson J (2004). Cell culture in drug discovery: an industrial perspective. In: van de Waterbeemd H, Lennernäs H, Artursson P, editors. Drug bioavailability. Weinheim: Wiley-Vch. p. 90–131.
16 Walle UK, Walle T (1998). Taxol transport by human intestinal epithelial Caco-2 cells. Drug Metab Dispos. **26**: 343–346.
17 Yee S (1997). In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man-fact or myth. Pharm Res. **14**: 763-766.