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Evaluation of a single-pass intestinal-perfusion method in rat for the prediction of absorption in man

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Abstract

Prediction of the fraction of dose absorbed from the intestine (F_a) in man is essential in the early drug discovery stage. In-vitro assays in Caco-2 and MDCK cells are routinely used for that purpose, and their predictive value has been reported. However, in-situ techniques might provide a more accurate estimation of F_a. In this study, we evaluated a single-pass intestinal-perfusion (SPIP) method in the rat for its use in the prediction of absorption in man and compared it with a previous report using cell-based assays. Effective permeability coefficients (P_{eff}) were determined in rats for 14 compounds, and ranged from 0.043×10^{-4} cm s^{-1} to 1.67×10^{-4} cm s^{-1}. These values strongly correlated ($r^2 = 0.88$) with reported P_{eff} values for man. In addition, the Spearman rank correlation coefficient calculated for in-situ-derived P_{eff} and absorption in man was 0.92 while for the previously tested in-vitro Caco-2 and MDCK systems vs absorption in man, the correlation coefficients were 0.61 and 0.59, respectively. SPIP provided a better prediction of human absorption than the cell-based assays. This method, although time consuming, could be used as a secondary test for studying the mechanisms governing the absorption of new compounds, and for predicting more accurately the fraction absorbed in man.

Introduction

The emergence of combinatorial chemistry has had a major impact on drug discovery, and has led to the development of high throughput screens to handle the increasing number of compounds and to predict their biopharmaceutical properties. Moreover, recent studies showed that inadequate pharmacokinetic properties were responsible for 39% of the withdrawals from development (Kennedy 1997). Among these defects, poor absorption was one of the causes for compound failure. Thus, predicting the fraction of oral dose absorbed has become very important in early drug discovery. Cell-based assays using Caco-2 and MDCK cell lines are commonly utilized for assessing the intestinal permeability of drug-discovery compounds (Garberg et al 1999; Stevenson et al 1999). These methods are well suited for high throughput screening and have been shown to produce permeability values which correlate with intestinal absorption in man (Artursson & Karlsson 1991; Rubas et al 1993; Irvine et al 1999). However, these cell lines are mostly used to predict passive absorption, and the results obtained are greatly affected by experimental parameters such as pH and co-solvents (Pauletti et al 1998; Yamashita et al 2000). In contrast, in-situ approaches provide experimental conditions closer to what is encountered following oral administration, with a lower sensitivity to pH variations due to a preserved microclimate above the epithelial cells

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(Hogerle & Winne 1983; Shiau et al 1985). These techniques maintain an intact blood supply to the intestine, and can be used to estimate the impact of clearance pathways, such as enzymes and transporters, that are present in the gut. Moreover, drug permeability (Ungell et al 1998) and expression of drug metabolizing enzymes and transporters have been shown to vary along the intestinal tract (Hakkak et al 1993; Zhang et al 1996; Makhey et al 1998), which can be investigated using intestinal perfusion of the various regions. In addition, oral drug absorption in rats was recently reported (Chiou & Barve 1998) to correlate very closely with that obtained in man. Thus, it is likely that the intestinal perfusion conducted in rats will give a better prediction of the fraction of oral dose absorbed in man than the in-vitro models.

In this study, we evaluated a single-pass intestinal-perfusion (SPIP) model and calculated the effective permeability of 14 compounds. These values were compared with those previously published in rats and man. We also determined whether SPIP in the rat offered a more accurate prediction of intestinal absorption in man than the cell-based assays.

Materials and Methods

Chemicals

Aciclovir and cefalexin were purchased from US Pharmacopeia (Rockville, MD) and HPLC-grade acetonitrile and trifluoroacetic acid (TFA) were from J-T Baker (Phillipsburg, NJ). All other chemicals were from Sigma Chemical Co. (St Louis, MO).

Animals and surgery

Male Sprague-Dawley rats (280–300 g), obtained from B&K (Fremont, CA), were maintained on a 12-h light–dark cycle and fasted 12–18 h before each experiment. They were anaesthetized by an intraperitoneal injection of Inactin (120 mg kg^{-1}) and placed on a heated pad to maintain normal body temperature.

A midline incision was made on the abdomen and an ileal segment of approximately 8–12 cm was isolated, using the ileo-caecal junction as a distal marker (Figure 1). Semi-circular incisions were made at each end using an electrocautery, and the lumen was rinsed with saline (37°C). Both ends were cannulated with PE tubing (PE205, 2.08 mm o.d.) and ligated using silk suture.

Blank perfusion buffer (see below) was first infused for 5 min at a flow rate of 1 mL min^{-1} by a syringe pump (Model 22, Harvard Apparatus, Holliston, MA), followed by perfusion of the compounds studied at a constant flow rate of 0.2 mL min^{-1} for 90 min. After cannulation, the segment was covered with isotonic saline-wet gauze (37°C). The perfusate was collected every 10 min. At the end of the perfusion, the intestinal segment was rinsed for 5 min with blank perfusion buffer (1 mL min^{-1}) and the length of the segment was measured following the last collection. Vials were weighed before and after collection, and were stored at -20°C until analysis. Approval for the studies was granted by the Institutional Animal Care and Use Committee at Affymax.

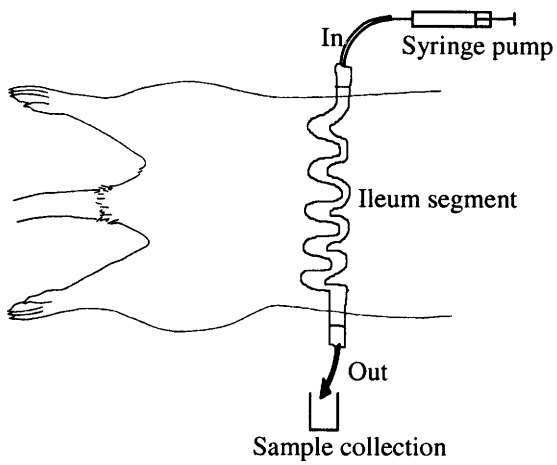

Figure 1 Intestinal perfusion set-up.

Composition of perfusion solutions

The perfusion buffer composition was as follows (mM): 138 NaCl, 2.7 KCl, 8.1 Na_2HPO_4, 1.5 KH_2PO_4. Phenol red (50 mg L^{-1}) was added to the solution as a non-absorbable marker. Antipyrine (1.05 mM) was included in each perfusion as a reference probe for passive absorption. Each compound was perfused separately, in association with phenol red and antipyrine. The pH was adjusted to 7.4 and the osmolality, measured by the freezing point depression method, was 290–310 mOsm kg^{-1} (Osmette A, Precision Systems Inc., Natick, MA). Preliminary experiments showed that no adsorption of the compounds on the catheters and the tubing took place.

Analytical methods

Samples were analysed by reverse-phase HPLC (HP1100 system, Agilent, Palo Alto, CA) using a Zorbax SB-C18 (3.0 × 75 mm) column and a diode array detector. The

mobile phase consisted of acetonitrile (0.01 % TFA)–water (0.01 % TFA). Specific gradients were used depending on the compounds analysed. Detection wavelengths for phenol red, sulfasalazine and aciclovir were 430 nm, 359 nm and 275 nm, respectively. Absorbance for the other compounds was measured at 230 nm.

Data analysis

Effective permeability coefficients (P_{eff}) were calculated from the steady-state concentrations of compounds in the perfusate collected at the distal end of the isolated intestinal segment. Steady state, which was assessed by a constant concentration of phenol red, was reached 30–40 min after the beginning of the experiment. P_{eff} was calculated using equation 1, according to the parallel-tube model (Komiya et al 1980; Levitt et al 1988).

$$P_{eff} = [-Q_{in} \cdot \ln (C_{out}/C_{in})]/2\pi rL \quad (1)$$

where C_{out} and C_{in} correspond to the outlet (corrected for volume changes) and inlet compound concentrations, respectively, while r is the intestinal radius (0.18 cm) (Komiya et al 1980) and L (cm) is the length of the isolated segment. Q (0.2 mL min^{-1}) is the flow rate through the intestinal segment.

The Spearman rank correlation coefficient, which measures the association derived from the ranks of two sets of observations, was calculated according to equation 2.

$$r_s = 1-(6\Sigma d^2)/(n^3-n) \quad (2)$$

where d is the difference of the two ranks associated with each compound, and n is the number of compounds (Fisher & Van Belle 1993).

Results and Discussion

Steady-state values of the effective permeability (P_{eff}) were determined in rats, in-situ, for 14 compounds using a SPIP model. The compounds selected presented various physicochemical properties and extents of absorption (Tables 1 and 2). They were chosen with the objective of relating our perfusion technique to that of Fagerholm et al (1996), and also to compare its predictive value for intestinal absorption in man with that of two cell lines previously tested (Irvine et al 1999). Compounds whose absorption is affected by active transporters were purposely included in the set and bioavailability data were not used since these might be complicated by metabolic factors. The P_{eff} values obtained were ranked and their correlation with intestinal absorption in man was compared with that achieved using Caco-2 and MDCK cell lines (Irvine et al 1999). Previous studies have examined the permeability of various compounds using single-pass intestinal-perfusions in the jejunum (Fagerholm et al 1996) and the ileum (Fagerholm et al 1997). P_{eff} of the compounds tested were not found to be statistically different in the ileum and the jejunum, although slightly lower in the ileum. These observations, as well as surgical convenience, led us to conduct our experiments in the ileum.

Equilibrium was reached within 30–40 min, as assessed by the concentration of phenol red in the outlet. The recovery of phenol red was 98.3 ± 1.0 %, in agreement with previous reports (Miller et al 1987), indicating that the intestinal mucosa was not damaged during the procedure. P_{eff} of antipyrine, which was used as a marker for passive absorption and as an indicator of major changes in mesenteric blood flow (Schulz & Winne 1987), was stable throughout the perfusion (CV < 10 %), confirming that no major physiological changes were occurring during the experiment. P_{eff} for antipyrine (0.73×10^{-4} cm s^{-1}) was similar to that recently determined (Lindahl et al 1998; Svensson et al 1999) but lower than previously reported values in the jejunum (Fagerholm et al 1996) and the ileum (Fagerholm et al 1997). Nevertheless, the rank order was consistent with the studies cited above when comparing the same subset of compounds (Table 2).

The P_{eff} values ranged from 0.043×10^{-4} cm s^{-1} to 1.67×10^{-4} cm s^{-1} (Table 3) and showed a high correlation ($r^2 = 0.88$) with those in man (Figure 2). Similarly, a strong correlation was recently observed between rats and man for the fraction of oral dose absorbed for a larger set of drugs (Chiou & Barve 1998) and for paracellularly absorbed compounds (He et al 1998). In Figure 2, a comparison of rat P_{eff} and intestinal absorption (F_a) in man showed that P_{eff} values greater than 0.5×10^{-4} cm s^{-1} corresponded to $F_a \sim 100$ % while P_{eff} values smaller than 0.2×10^{-4} cm s^{-1} corresponded to F_a values lower than 70 %.

The rank order for the P_{eff} values in rat was compared with that of F_a in man and the Spearman rank correlation coefficient (r_s), calculated for SPIP and F_a, was 0.92 ($P < 0.01$, Table 4). In contrast, when F_a was compared with the apparent permeability (P_{app}) from cell-based assays, r_s was only 0.61 and 0.59 for Caco-2 and MDCK cells, respectively.

The higher predictive value obtained from the in-situ experiments is not unexpected; the SPIP model provides conditions close to a normal physiological state, with an

Table 1 Physicochemical properties and concentrations used for the compounds tested in the single-pass intestinal-perfusion.

Compound	Molecular weight (g mol^{-1})	pK$_a$	log D$_{oct}$ pH 7.4	C$_{in}$ (mM)
Aciclovir	225	2.3, 9.3[a]	−0.3[b]	0.20
Antipyrine	188	1.5[c]	0.4[c]	1.05
Atenolol	266	9.6[c]	−1.8[c]	0.83
Cefalexin	347	5.2, 7.3[d]	−1.0[e]	0.10
Dexamethasone	392	*	2.1[b]	0.10
Furosemide	331	3.8[c]	−0.8[c]	0.20
Hydrochlorothiazide	297	8.8, 10.1[f]	−0.2[f]	0.40
Ketoprofen	254	4.6[c]	0.3[c]	0.67
Metoprolol	267	9.7[c]	0.0[c]	0.58
Nadolol	309	9.7[a]	0.6[b]	0.60
Naproxen	230	4.4[c]	0.1[c]	1.8
Phenol red	354	*	*	0.05 mg mL^{-1}
Propranolol	259	9.5[f]	1.4[f]	0.60
Sulfasalazine	398	2.4, 9.7, 11.8[a]	−0.4[b]	0.30
Terbutaline	225	8.8, 10.1, 11.2[c]	−1.4[c]	0.10

[a]Taken from Ren & Lien (2000). [b]Taken from Yazdanian et al (1998). [c]Taken from Fagerholm et al (1997). [d]Taken from Gennaro (1995). [e]Taken from Kansy et al (1998). [f]Taken from Winiwarter et al (1998). *No information available. C$_{in}$, inlet compound concentration.

Table 2 Intestinal permeability coefficients determined in rats and man.

Compound	P$_{eff}$ (10^{-4} cm s^{-1})		Rank order (rat/man)
	Rat, experimental	Man, literature[a]	
Antipyrine	0.73 ± 0.02	5.6 ± 1.6	7/7
Atenolol	0.18 ± 0.09	0.15 ± 0.20	3/2
Furosemide	0.19 ± 0.03	0.3 ± 0.3	4/3
Hydrochlorothiazide	0.07 ± 0.03	0.04 ± 0.05[b]	1/1
Ketoprofen	1.09 ± 0.71	8.5 ± 3.9	8/9
Metoprolol	0.59 ± 0.13	1.5 ± 0.9	5/5
Naproxen	1.67 ± 0.82	8.0 ± 4.2	9/8
Propranolol	0.66 ± 0.29	2.8 ± 1.3[c]	6/6
Terbutaline	0.12 ± 0.09	0.3 ± 0.3	2/3

The rat P$_{eff}$ (effective permeability coefficient) values are reported as the mean ± s.d. of at least three independent experiments. [a]Taken from Fagerholm et al (1996). [b]Taken from Winiwarter et al (1998). [c]Taken from Lennernas et al (1997).

intact blood supply and a functional intestinal barrier. However, it is apparent from Figure 3 that the rat P$_{eff}$ of cefalexin underestimates its F$_a$ in humans. The F$_a$ of cefalexin was also poorly predicted by transport studies across Caco-2 and MDCK cells (Chong et al 1996; Irvine et al 1999). Cefalexin is a substrate for the dipeptide transporter (PepT1), whose expression has been reported in Caco-2 (Dantzig & Bergin 1990) and MDCK cells (Ganapathy et al 1995). A possible explanation for the low P$_{app}$ value obtained in these cell lines (Irvine et al 1999) is that no proton gradient was applied, reducing the involvement of the transporter. The discrepancy between P$_{eff}$ and F$_a$ may result from a low expression of PepT1 in the rat ileum compared with human intestine, leading to lower absorption, or from saturation of the transporter at the concentration used in our studies. However, the contribution of PepT1 to the absorption of SQ-29852, a probe substrate, was found to be constant throughout the rat intestine (Marino et al 1996). These results were also consistent

Table 3 Absorption parameters of 14 compounds determined in MDCK and Caco-2 cell lines, in rat intestinal perfusion and in man.

Compounds	Rat P_{eff} (10^{-4} cm s^{-1})	MDCK P_{app} [a] (10^{-4} cm s^{-1})	Caco-2 P_{app} [a] (10^{-4} cm s^{-1})	Absorption in man[b] (%)
Aciclovir	0.07±0.02	0.02±0.02	Not detected	16[c]
Antipyrine	0.73±0.02	1.50±1.00	1.50±0.12	100
Atenolol	0.18±0.09	0.018±0.009	0.033±0.004	50
Cefalexin	0.26±0.06	0.005±0.002	0.003±0.001	98
Dexamethasone	1.57±0.21	0.20±0.02	0.40±0.04	100
Furosemide	0.19±0.03	0.006±0.004	0.0014±0.0001	61
Hydrochlorothiazide	0.07±0.03	0.010±0.003	0.09±0.04	67
Ketoprofen	1.09±0.72	0.20±0.02	0.93±0.10	100
Metoprolol	0.59±0.13	1.50±0.10	1.40±0.10	95
Nadolol	0.043±0.007	0.01±0.04	0.004±0.003	34
Naproxen	1.67±0.82	N.D.	N.D.	100
Propranolol	0.66±0.29	1.70±0.06	1.10±0.13	90
Sulfasalazine	0.06±0.03	0.005±0.004	0.006±0.011	13[d]
Terbutaline	0.12±0.09	0.010±0.003	0.004±0.002	60[e]

The rat effective permeability coefficient (P_{eff}) values are reported as the mean±s.d. of at least three independent experiments. [a]Taken from Irvine et al (1999). [b]Taken from Wessel et al (1998). [c]Obtained from Glaxo Wellcome. [d]Taken from Artursson & Karlsson (1991). [e]Taken from Fagerholm et al (1996). N.D., parameter not determined; P_{app}, apparent permeability.

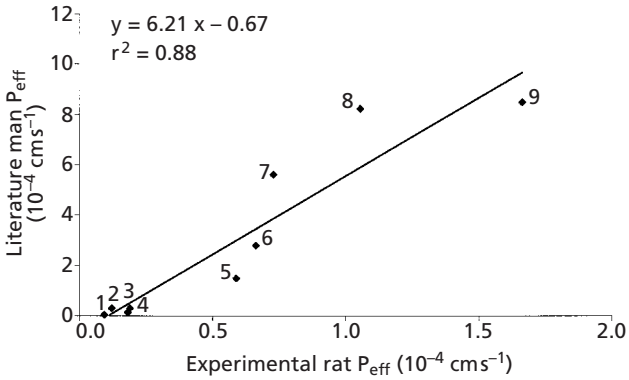

Figure 2 Comparison between effective intestinal permeability coefficients (P_{eff}) in man and rat for 9 of the 14 compounds tested. P_{eff} values in man were taken from Fagerholm et al (1996), Lennernas et al (1997) and Winiwarter et al (1998). 1, Hydrochlorothiazide; 2, terbutaline; 3, atenolol; 4, furosemide; 5, metoprolol; 6, propranolol; 7, antipyrine; 8, ketoprofen; 9, naproxen.

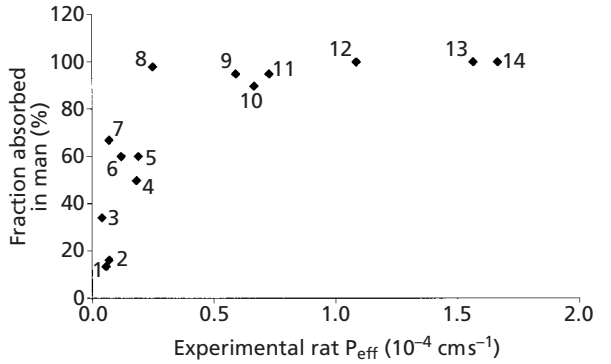

Figure 3 Fraction of drug dose absorbed in man (F_a) vs rat P_{eff} value. F_a values were taken from Wessel et al (1998), Artursson & Karlsson (1991) and Fagerholm et al (1996), and obtained from Glaxo Wellcome. 1, Sulfasalazine; 2, aciclovir; 3, nadolol; 4, atenolol; 5, furosemide; 6, terbutaline; 7, hydrochlorothiazide; 8, cefalexin; 9, metoprolol; 10, propranolol; 11, antipyrine; 12, ketoprofen; 13, dexamethasone; 14, naproxen.

Table 4 Spearman rank correlation coefficients.

Comparison	r_s
Rat perfusion vs absorption in man	0.92 (n = 14)
MDCK vs absorption in man	0.59 (n = 13)[a]
Caco-2 vs absorption in man	0.61 (n = 13)[a]

[a]Adapted from Irvine et al (1999).

with studies reporting similar PepT1 mRNA (Miyamoto et al 1996) and protein levels (Ogihara et al 1996) in the rat duodenum, jejunum and ileum. In addition, the K_m for cefalexin transport by rat PepT1 was 7.2 mM (Sinko & Amidon 1988), two orders of magnitude higher than the concentration used in our studies (100 μM), ruling out saturation of the transporter. The pH of the perfusion solution was adjusted to 7.4, matching the physi-

ological pH in the ileum (Kararli 1995); this may also have affected the PepT1-mediated absorption of cefalexin. Our studies, however, were not designed to examine in detail the mechanisms involved. Interestingly, the intestinal absorption of cefalexin was also slightly underestimated by the wall permeability, P_W, calculated from a perfusion in rat ileum (Amidon et al 1988).

Rat ileal P_{eff} estimates obtained in this study showed a high correlation with P_{eff} values previously reported in man and rat, confirming the validity of our procedure. The in-situ technique used here also provided a greater correlation with intestinal absorption in man than did Caco-2 and MDCK cell lines. Although time consuming and not adapted for high throughput screening, SPIP could be utilized as a complementary absorption test for new chemical entities, and could help determine the involvement of intestinal enzymes and transporters in absorption. This technique can also be applied to the development of computational models for more accurately predicting and confirming site-specific absorption (Burton 2000).

Finally, these studies show some of the limitations of the current cell-based assays, and emphasize the need for more predictive high throughput in-vitro and in-silico models. Ongoing work in our laboratory is focusing on the development of such models using the results from the perfusion method described.

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