Research Paper

In Situ Single-Pass Intestinal Perfusion in Rats for Intestinal Permeability Investigation of Drugs

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The aim of this study was to establish the *in situ* single-pass intestinal perfusion method in rats for intestinal permeability investigation of drug. Effective permeability coefficients (P_{eff}) of 15 model drugs were investigated in rat jejunal. Krebs-Ringer buffer solution containing drug was perfused at flow rate of 0.2 ml/min and samples were taken from outlet up to 110 min, steady state was achieved after 30 min perfusion without samples taken. Gravimetric method was used to correct the net water flux. Drug concentrations in perfusion samples were determined using high-performance liquid chromatography-ultraviolet method and P_{eff} were calculated. The P_{eff} values obtained by *in situ* single-pass intestinal perfusion ranged from $0.0146\pm0.0026\times10^{-4}$ cm/s of enalaprilat to $1.77\pm0.34\times10^{-4}$ cm/s of antipyrine. Among the selected model drugs, the P_{eff} values of high permeability drugs were higher than 0.2×10^{-4} cm/s, and less than 0.03×10^{-4} cm/s for low permeability drugs. The experimental rat P_{eff} was highly correlated to the literature human P_{eff} with correlation coefficient (R^2) of 0.99. The observed experimental rat P_{eff} values were highly correlated to the literature human F_{abs} (R^2=0.93). All investigated model drugs have similar permeability classification compare with literature permeability classification. The predicted human F_{abs} were linearly correlated with literature human F_{abs} (R^2=0.90). This method could be used for investigation of the intestinal absorption of new compounds, and for prediction the absorption fraction in human. Besides, investigation of the permeability classification of generic drug by *in situ* single-pass intestinal perfusion can be used for application of biowaiver for industry.

Key words: Biopharmaceutics classification system, intestinal permeability, *in situ* single-pass intestinal perfusion, effective permeability coefficient

The Biopharmaceutics Classification System (BCS), first proposed by Amidon *et al.*[1] in 1995, is a scientific frame for classifying drugs according to solubility of the drug dose in the Gastrointestinal (GI) milieu and permeability of the drug through the GI membrane. Drugs are divided into 4 categories according to BCS: BCS class I (high solubility-high permeability), BCS class II (low solubility-high permeability), BCS class III (high solubility-low permeability) and BCS class IV (low solubility-low permeability). Before the BCS classification system was proposed, it was difficult for industry to find theoretical support to apply biowaiver from drug regulatory department. Based on this frame, when an immediate-release solid oral dosage form drug with a rapid dissolution compare to gastric emptying and the drug has high solubility, the absorption of drug is most likely to be dependent on the permeability of drug on intestine membrane. Therefore, it is not necessary for drug products containing BCS I and BCS III active ingredients to prove the *in vivo* bioavailability and bioequivalence, as long as the absorption of active ingredients was not significantly affected by the excipients used in the dosage form[2,3].

Solubility parameters of drugs are readily available compared to permeability parameters. The model for evaluating permeability parameters of drugs

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has two major categories, *in vivo* and *in vitro*. *In vivo* models include absorption studies in humans and animals; *in vitro* models include Parallel Artificial Membrane Permeability Assay, tissue model (Ussing Perfusion Chamber model), *in situ* model (Intestinal Perfusion model) and cell model. Among all the permeability study models, the data from *in vivo* model can truly reflect the absorption of drugs in the body system but it is difficulty to get. Rat *in situ* intestinal perfusion have become the most reliable and cost-effective model in all permeability study model. Perfusion studies in rat intestine were highly correlated with human data, even though the type of transporters and their expression levels may vary between species[4,5]. It has been reported that a high correlation was observed between human and rat small intestine permeability (R^2=0.80-0.95) for drugs which permeate through the intestinal by carrier-mediated absorption and passive diffusion mechanisms[6].

In situ Single-Pass Intestinal Perfusion (SPIP) model provides the advantages of precise experimental control, intact blood supply, and ability to investigate regional factors influencing intestinal absorption of compounds[7-9]. It has been widely utilized to predict the extent of drug absorption and to clarify absorption mechanisms[6,10]. A list of 15 drugs, which proposed as model drugs by Food and Drug Administration (FDA) for investigation intestinal permeability, were chosen with different permeability characteristics: low, moderate and high permeability. In this work, we investigated the effective permeability coefficients (P_{eff}) of these model drugs and compared with literature human data to establish the study model for intestinal permeability of drug.

MATERIALS AND METHODS

Materials:

Enalaprilat, lisinopril, amiloride, aciclovir, phenytoin, propranolol, metoprolol, ketoprofen, chlorothiazide, ranitidine, atenolol, hydrochlorothiazide, terbutaline, metformin and antipyrine were purchased from National Institutes for Food and Drug Control (Beijing, China). Acetonitrile, methanol (High-Performance Liquid Chromatography (HPLC) grade) were obtained from TEDIA (Ohio, USA). Deionized water was generated by Milli-Q system from Millipore (Bedford, USA). All other chemicals were analytical reagent grade.

In situ single-pass intestinal perfusion in rat:

All experimental protocols were supervised and managed by Jiangsu Provincial Drug Safety Evaluation Center Agency Committee (protocol code: JA-18-009) and the experiments were conducted in accordance with the guide for the related laws and regulations and Institutional Animal Care and Use Committee (IACUC). Male Sprague Dawley rats weighing 250-350 g (Zhejiang Vital River Laboratory Animal Technology Co., Ltd., Zhejiang, China) were maintained on a 12 h light-dark cycle and had free access to water and food, six replicates for each drug. Rats were fasted for 12 h (water *ad libitum*) prior to each experiment, and then anaesthetized with pentobarbital solution (30 mg/kg, i.p.). Then placed on a heated slide warmer and under a heating lamp to maintain normal body temperature. The abdomen was opened with a midline incision and jejunal segment of approximately 10 cm was measured, isolated and cannulated with plastic tubing[11-13]. Cares was taken to avoid disturbance of the circulatory system and the exposed segment was kept moist with 37° saline. Initially, the intestinal segment was rinsed with isotonic saline (37°) until the outlet solution was clear. Krebs-Ringer buffer solution, consisted of 130 mM NaCl, 5mM KCl, 1.27 mM $MgSO_4$, 0.95 mM $CaCl_2$, 5 mM glucose and 10 mM NaH_2PO_4, was used as blank perfusion solution. Perfusion solution was prepared by dissolving drugs in Krebs-Ringer buffer solution and then perfused through the intestinal segment at a flow rate of 0.2 ml/min. Blank perfusion solution was perfused for 30 min without sample taken to ensure steady state conditions, followed by additional 80 min of perfusion with samples taken every 20 min. Gravimetric method was used to correct the Net Water Flux (NWF)[14-17]. All samples were centrifuged at 16 000 rpm for 2 min before analyzed by HPLC. The length of the perfused intestinal segment was measured at the endpoint of the experiment.

The P_{eff} (cm/sec) of model drugs through the rat gut wall was determined according to the following equations:

$$C_{out\ (corrected)}=C_{out}Q_{out}/Q_{in} \quad (1)$$

$$P_{eff}=-Q\ln(C_{out\ (corrected)}/C_{in})/2\pi RL \quad (2)$$

Where C_{out} and C_{in} are the outlet and the inlet concentration of drug respectively, Q_{out} and Q_{in} are the volume that collected at the outlet and the volume that perfusion solution reduced at the inlet

respectively, $C_{out(corrected)}$ is the outlet concentration of drug that has been corrected NWF by Gravimetric method, Q is the perfusion buffer flow rate (0.2 ml/min), R is the radius of the intestinal segment, and L is the length of the perfused intestinal segment.

Analytical methods:

All *in situ* SPIP samples were quantified by a Essentia LC-15C HPLC (Shimadzu, Japan). More details about analytical methods of each model drug can be find in supporting information. Processing and analysis of chromatogram were performed on LabSolutions Essentia software.

Statistical analysis:

Values are expressed as mean±Standard Deviation (SD). The independent t test and one-way Analysis of Variance (ANOVA) were used to assess differences comparison of permeation parameter. Differences were considered statistically significant when $p<0.05$. The statistical comparison was made using the statistical package SPSS, V.23.

RESULTS AND DISCUSSION

Permeability values of model drugs were investigated by *in situ* SPIP in rat at steady-state. During the perfusion, the collected sample were centrifuged, and then injected into the HPLC system for analysis. Gravimetric method was used to correct the NWF of rat intestine during the perfusion process. The corrected outlet concentration of each drug was obtained from equation (1), P_{eff} value was calculated from equation (2). The permeability values were presented in fig. 1, and summarized in Table 1. The P_{eff} of different model drugs ranged from $0.0146\pm0.0026\times10^{-4}$ cm/s for enalaprilat and $1.77\pm0.34\times10^{-4}$ cm/s for antipyrine, respectively.

During perfusion, the intestine will absorb and secrete water, which affects the calculation of the P_{eff}. It is necessary to correct the NWF. NWF correction during the SPIP perfusion was usually involves the co-perfusion of a "non-absorbed" marker[14]. Since phenol red is an ionic compound, it is conventionally considered to be not absorbed in the intestine, and thus it has long been used as a correcting substance for correcting the NWF. However, it has been reported in the literature that phenol red is absorbed in the intestine[18]. For drugs with high permeability, the influence caused by phenol red has little effect on the test results, which is negligible, but for drugs with low permeability, the influence caused by phenol red cannot be ignored. Therefore, gravimetric method was used to correct the NWF during the perfusion in this study.

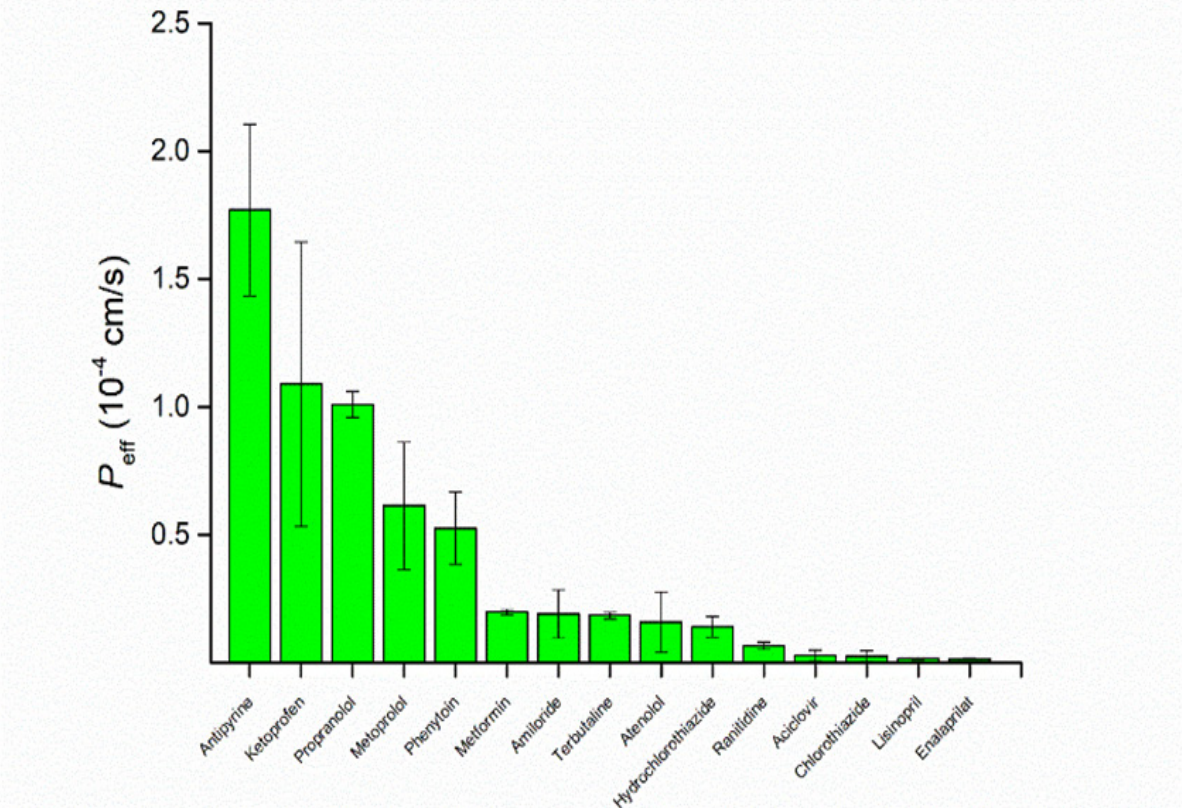

Fig. 1: The permeability values of 15 different model drugs obtained by *in situ* SPIP method (mean±SD, n=6)

TABLE 1: THE PERFUSION CONCENTRATIONS AND PERMEABILITY VALUES OF DIFFERENT MODEL DRUGS OBTAINED BY *IN SITU* SPIP METHOD (MEAN±SD, n=6)

Drug	Concentration (μg/ml)	P_{eff} (10^{-4} cm/s)
Antipyrine	120	1.77±0.34
Ketoprofen	120	1.09±0.56
Propranolol	120	1.01±0.05
Metoprolol	120	0.613±0.249
Phenytoin	30	0.526±0.141
Metformin	120	0.198±0.0114
Amiloride	120	0.190±0.094
Terbutaline	120	0.185±0.014
Atenolol	120	0.159±0.117
Hydrochlorothiazide	120	0.140±0.041
Ranitidine	120	0.0668±0.0147
Aciclovir	120	0.0282±0.0208
Chlorothiazide	120	0.0254±0.0212
Lisinopril	120	0.0161±0.0028
Enalaprilat	120	0.0146±0.0026

In this work, the P_{eff} values investigated by *in situ* SPIP in rat were ranged from $0.0146\pm0.0026\times10^{-4}$ cm/s for enalaprilat to $1.77\pm0.34\times10^{-4}$ cm/s for antipyrine. Drug with $P_{eff}<0.03\times10^{-4}$ cm/s in the rat small intestine is classified as low permeability, whereas drug with $P_{eff}>0.2\times10-4$ cm/s is completely absorbed (classified as high permeability)[4]. Among the selected model drugs, the P_{eff} of high permeability drugs obtained in this experiment were higher than 0.2×10^{-4} cm/s and P_{eff} of low permeability drugs were less than 0.03×10^{-4} cm/s, indicating a high correlation of *in situ* SPIP model to permeability classification of drug. For industry with desire in generic drug development, the investigation in permeability of drugs by a appropriate method is crucial. The FDA has approved the biowaiver for immediate-release solid oral dosage forms based on a BCS system, which divided drugs in different categories according to solubility and permeability. The *in situ* SPIP method could be suitable for investigation of permeability.

Table 2 summarizes the permeability values for 8 different model drugs obtained by in-situ SPIP and literature human P_{eff} data[19-22]. The correlation between the two sets of data, the *in situ* SPIP *vs.* human P_{eff} value, was presented in fig. 2. It can be seen that an excellent correlation was obtained between the experimental rat P_{eff} and literature human P_{eff}, as evident by correlation coefficient (R^2) of 0.99, which indicating the validity of this work. These results indicated that *in situ* SPIP method could be used for investigation of the intestinal absorption of new compounds, and for predicting the absorption fraction in human, which is important in early drug development.

A strong correlation was observed between experimental rat P_{eff} data and literature human F_{abs} (R^2=0.93). Fig. 3 fitting to the follow equation;

$$F_{abs}=1-e^{-7.06Peff} \quad (3)$$

Table 3 summarizes the literature human F_{abs} data, and the corresponding predicted F_{abs} by the experimental model using above-mentioned equation, and BCS permeability class[19,23]. According to FDA, when there is no evidence to suggest the instability in gastrointestinal tract, drugs can be considered as high permeability drug when the fraction absorbed in human gastrointestinal tract is investigated to be 85 % or more[2]. This means that drugs with F_{abs} higher than 85 % can be considered as high permeability drug. As shown in Table 3, F_{abs} of 5 different model drugs obtained by equation (3) were higher than 85 %, indicating a high correlation of absorption fraction between this work and literature. It can be seen in Table 3 that the F_{abs} of metformin was 86 %, which was very nearly to the boundary between high permeability and moderate permeability. It was

classed as moderate permeability by FDA. Similar situation have occurred in ranitidine, which literature F_{abs} was 50 %, nearly to the boundary between moderate permeability and low permeability. Similar finding also have been found in other literature[6]. In this work, the predicted F_{abs} of metformin and ranitidine using above-mentioned equation was 75 % and 38 %, respectively. When drugs with F_{abs} nearly to the boundary between different permeability classification, the application of the *in situ* SPIP model may not distinguish them well, which may be a limitation of the model. In this study, all the investigated model drugs by *in situ* SPIP have similar permeability classification compare with literature permeability classification. The predicted human F_{abs} by *in situ* SPIP model were linearly correlated with literature human F_{abs} (R^2=0.90) (fig. 4).

TABLE 2: PERMEABILITY VALUES FOR 8 OF 15 DIFFERENT MODEL DRUGS OBTAINED BY *IN SITU* SPIP AND LITERATURE HUMAN P_{eff} DATA. DATA PRESENTED AS MEAN±SD

Drug	P_{eff} (10^{-4} cm/s)	
	Human, literature	Rat, experimental
Antipyrine	5.6±1.6	1.77±0.34
Propranolol	2.8±1.3	1.01±0.05
Metoprolol	1.5±0.9	0.613±0.249
Terbutaline	0.3±0.3	0.185±0.014
Atenolol	0.15±0.2	0.159±0.117
Hydrochlorothiazide	0.04±0.05	0.140±0.041
Ranitidine	0.273±0.247	0.0668±0.0147
Enalaprilat	0.1±0.3	0.0146±0.0026

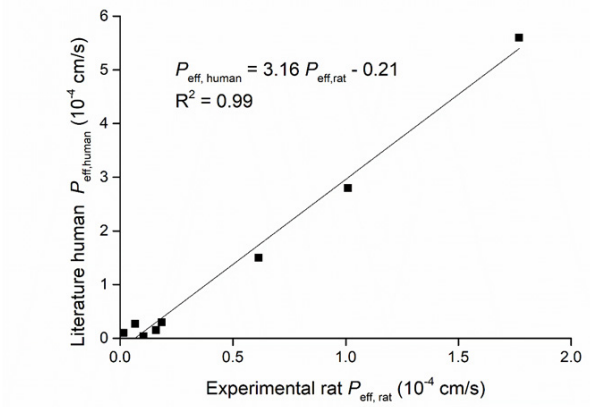

Fig. 2: The correlation between literature human P_{eff} and experimental rat P_{eff} for 8 of 15 different model drugs

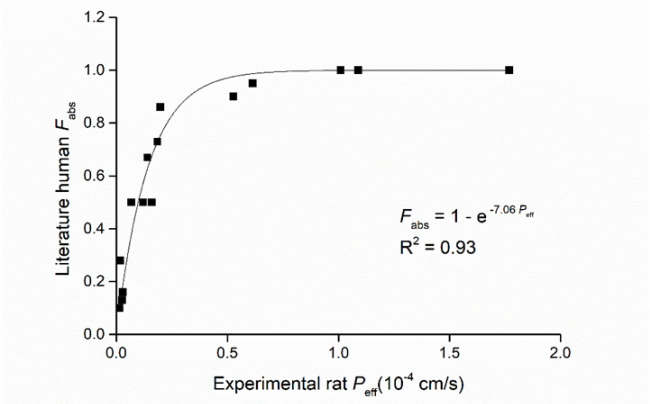

Fig. 3: The correlation between literature human F_{abs} and experimental rat P_{eff} of 15 different model drugs

TABLE 3: LITERATURE HUMAN F_{abs} DATA AND CORRESPONDING PREDICTED F_{abs} AND BCS PERMEABILITY CLASS OF 15 DIFFERENT MODEL DRUGS

Drug	Literature human data		Predicted data	
	F_{abs} (%)	Permeability class	F_{abs} (%)	Permeability class
Antipyrine	100	H	100	H
Ketoprofen	100	H	100	H
Propranolol	100	H	100	H
Metoprolol	95	H	99	H
Phenytoin	90	H	98	H
Metformin	86	M	75	M
Amiloride	50	M	74	M
Terbutaline	73	M	73	M
Atenolol	50	M	67	M
Hydrochlorothiazide	67	M	63	M
Ranitidine	50	M	38	M
Aciclovir	16	L	18	L
Chlorothiazide	13	L	16	L
Lisinopril	28	L	11	L
Enalaprilat	10	L	10	L

Note: H: High; M: Moderate and L: Low

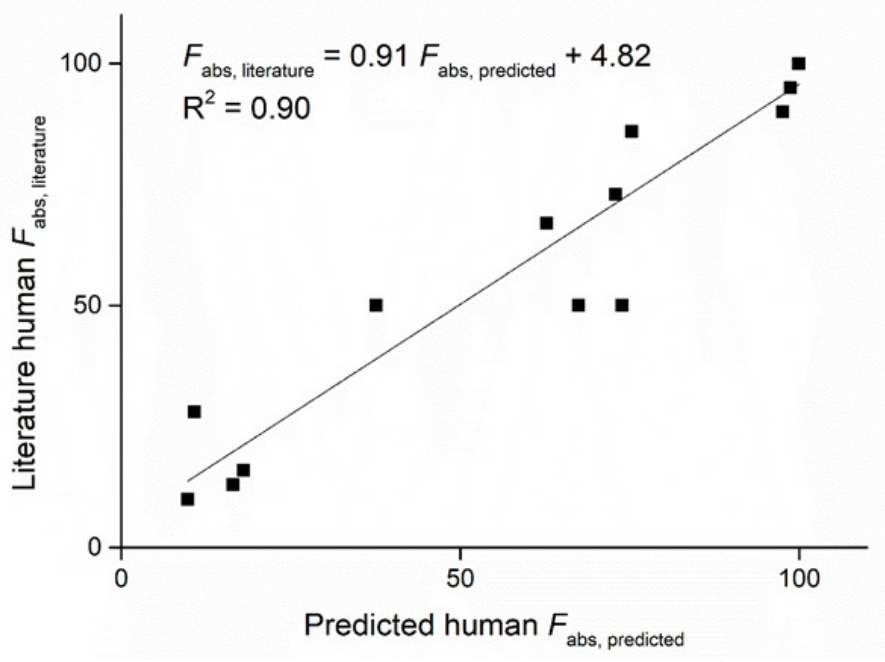

Fig. 4: The correlation between literature human F_{abs} and predicted human F_{abs} of 15 different model drugs

In this work, we investigated the P_{eff} of 15 model drugs with different permeability classification by *in situ* SPIP method. The P_{eff} of high permeability drugs were higher than 0.2×10^{-4} cm/s and P_{eff} of low permeability drugs were less than 0.03×10^{-4} cm/s. The P_{eff} values obtained in this work showed a high correlation with those in human, and a strong correlation was observed between human F_{abs} and experimental rat P_{eff}. Besides, the literature human F_{abs} and predicted human F_{abs} was strongly correlated. The results indicating that *in situ* SPIP method could be used for investigation of the intestinal absorption of new compounds and for prediction the absorption fraction in human, which is crucial in drug development.

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Conflict of interests:

The authors declare that they have no competing interest.

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