International Journal of Pharmaceutics 551 (2018) 148–157

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Determination of intestinal permeability using *in situ* perfusion model in rats: Challenges and advantages to BCS classification applied to digoxin

Tamires Guedes Caldeira[a,b,1], Alejandro Ruiz-Picazo[a,1], Isabel Lozoya-Agullo[a], Dênia Antunes Saúde-Guimarães[c], Marta González-Álvarez[a], Jacqueline de Souza[b], Isabel González-Álvarez[a,*], Marival Bermejo[a]

[a] Department of Engineering, Pharmaceutics and Pharmaceutical Technology Area, Universidad Miguel Hernández de Elche, Alicante, Spain
[b] Laboratório de Controle de Qualidade, Escola de Farmácia, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais, Brazil
[c] Laboratório de Plantas Medicinais, Escola de Farmácia, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais, Brazil

ARTICLE INFO

Keywords:
Intestinal absorption
In situ perfusion
Digoxin
Permeability
Biopharmaceutic Classification System

ABSTRACT

The purpose of this work was to describe the closed loop *in situ* perfusion method in rats and to compare the difficulties and advantages with other methods proposed by regulatory agencies for BCS classification and finally to illustrate its application to evaluate the permeability of digoxin at relevant clinical concentrations. Digoxin was evaluated at two concentration levels: 1.0 μg/ml (with and without sodium azide 65.0 μg/ml) and 6.0 μg/ml. These concentrations correspond to the ratio of the highest dose strength (0.25 mg) and the highest single dose administered (1.5 mg) and the 250 ml of water. *In situ* closed loop perfusion studies in rats were performed in the whole small intestine and also in duodenum, jejunum and ileum segments to evaluate the relevance of P-gp secretion in the overall permeability. A kinetic modelling approach involving passive permeation and efflux transport mechanism allowed the estimation of the passive diffusional component and the Michaelis-menten parameters. The estimated K_m value demonstrated that at clinical luminal concentrations the efflux process is not saturated and then it could be inhibited by other drugs, excipients or food components leading to the already reported clinical drug-drug and drug-food interations. The present data confirms from a mechanistic point of view these interactions.

1. Introduction

According to the European Medicines Agency (EMA), BCS (Biopharmaceutic Classification System) permeability classification of a drug requires absolute bioavailability or mass balance studies. However, the Food and Drug Administration (FDA) and World Health Organization (WHO) accept for BCS classification the results from other methods able to predict the extent of drug absorption in humans, such as *in vivo* or *in situ* intestinal perfusion in animal models (e.g. rats), or *in vitro* permeability methods using intestinal tissues or monolayers of suitable epithelial cells (EMA, 2010, FDA, 2015, WHO, 2015).

According to the BCS, solubility, permeability and dissolution rate from dosage form are the fundamental parameters that control drug absorption rate and extent. Based on this concept, drugs were grouped into four classes: class I, high solubility and high permeability, class II, low solubility and high permeability, class III, high solubility and low permeability and class IV, low solubility and low permeability (Amidon et al., 1995).

Among the different methods used to evaluate the permeability, *in situ* intestinal perfusion has been widely used due to its similarity with *in vivo* conditions such as the presence of blood irrigation, innervation, active transporters, metabolizing enzymes and compatibility with complex solvent systems (Balimane et al., 2000, Volpe, 2010, Stappaerts et al., 2015).

In addition, *in situ* perfusion in rats can also be used to investigate drug absorption in the presence of pathophysiological changes, for instance, changes in motility and intestinal barrier integrity associated with parasitic diseases. Fast and fed states can also be simulated in the perfusion method by changing the perfusion media (since there is a change in the pH and composition of intestinal fluids in postprandial conditions) (Lautenschläger et al., 2010, Stappaerts et al., 2015).

In situ intestinal perfusion in rats presents a good correlation with

* Corresponding author at: Pharmacokinetics and Pharmaceutical Technology, Department of Engineering, Carretera Nacional 332 Alicante-Valencia, Campus de San Juan Edificio 2. CP, 03550 San Juan de Alicante, Alicante, Spain.
E-mail address: isabel.gonzalez@goumh.umh.es (I. González-Álvarez).
[1] Both author are considered jointly first authors as they contributed equally to this work.

https://doi.org/10.1016/j.ijpharm.2018.09.022
Received 15 July 2018; Received in revised form 11 September 2018; Accepted 11 September 2018
Available online 13 September 2018
0378-5173/

human intestinal permeability, for passively absorbed drugs, and moderate correlation between the expression levels of transporters in the rat and human, which provides similar molecular mechanisms for the absorption of drugs between the two species. Therefore, the permeability of passively transported compounds can be predicted with a particularly high degree of accuracy. However, special care must be taken for drugs with a carrier-mediated transport mechanism, and a scaling factor has to be used (Lennernäs, 1997, Cao et al., 2006, Lennernäs, 2014, Lozoya-Agullo et al., 2015b).

Then, a well-established protocol for permeability studies using the *in situ* intestinal perfusion can clarify the transport mechanisms of a drug through the intestinal barrier, contributing to its adequate biopharmaceutical classification and correlating, in a reliable way, with the process of oral bioavailability and therapeutic action of the drug (Dezani et al., 2017).

Therefore, the purpose of this work is to describe the *in situ* closed-loop perfusion method in rats (Doluisio et al., 1969), and to discuss the difficulties and advantages in comparison with other methods proposed by regulatory agencies for BCS classification, illustrating its application to evaluate the permeability of digoxin as model drug. Digoxin is a cardiac glycoside with capacity to increase the contractile force of the myocytes due to positive inotropic effect. Digoxin has a narrow therapeutic index (NTI) and in order to obtain an adequate response and avoid toxicity, the oral dosage can vary from 0.25 mg to 1.5 mg, in patients with normal body weight and renal function (Neuhoff et al., 2013). It is a well-known substrate of P-glycoprotein (Gandia et al., 2004, Yao and Chiou, 2006, Suzuki et al., 2014, Nielsen et al., 2016, Oda and Murakami, 2017), but most of the permeability reported data in the literature have been obtained at concentrations that exceed the clinically relevant concentrations in lumen reflecting mainly the passive diffusional component of its membrane transport. Thus, in addition to demonstrating the applicability of the *in situ* closed-loop perfusion method in rats and determining the permeability of digoxin at clinically relevant concentrations, the saturation level of the transporter was evaluated, which could explain the reported drug-drug and drug-food reported interactions (Hanratty et al., 2000, Yao and Chiou, 2006).

2. Materials and methods

2.1. Materials

Digoxin and sodium azide were purchased from Sigma-Aldrich (St Louis, MO, USA). Acetonitrile HPLC grade was purchased from VWR Chemicals (Fontenay-sous-Bois, France). Water was obtained from a Millipore purification system. All other chemicals were of analytical grade or higher.

2.2. Perfusion solution

Perfusion solution used in the *in situ* intestinal perfusion studies was prepared with phosphate buffer 50 mM ajusted to pH 6.8.

Digoxin was evaluated at two concentration levels: 1.0 µg/ml (with and without sodium azide 65.0 µg/ml) and 6.0 µg/ml. These concentrations were obtained by dividing the highest dose strength (0.25 mg) and the highest single dose administered (1.5 mg) of digoxin that can be administered with 250 ml of water (volume of water generally used for the administration of a drug product in bioequivalence studies).

Perfusion solutions were maintained at 37 °C until use.

2.3. Animals

Male Wistar rats weighting 250–300 g (n = 4–6) were used for all *in situ* permeability studies. Animal experiments were approved by the Scientific Committee of the Faculty of Pharmacy, Miguel Hernandez University, and followed the guidelines described in the EC Directive 86/609, the Council of the Europe Convention ETS 123 and Spanish national laws governing the use of animals in research.

2.4. Intestinal perfusion of digoxin

Effective permeability of digoxin was determined in the complete small intestine at concentrations of 1.0 µg/ml and 6.0 µg/ml and in duodenum, jejunum and ileum only at the concentration of 1.0 µg/ml using *in situ* closed-loop perfusion based in Doluisio method (Doluisio et al., 1969) modified to evaluate in each intestinal segment (Lozoya-Agullo et al., 2016). In addition, the passive permeability of the drug was evaluated in the ileum with the perfusion solution of digoxin 1.0 µg/ml and sodium azide 65.0 µg/ml.

Rats were anesthetized with intraperitoneal injection of pentobarbital (40 mg/kg) and divided into two groups. In the first group, an abdominal midline incision was made and the complete small intestine (approximately 100 cm) was identified and cannulated at both ends using glass cannulae attached to silicone tubing for conection with stopcock valves and glass syringes. In the second group, segments of duodenum (approximately 10 cm), jejunum (approximately 45 cm) and ileum (approximately 45 cm) were identified and cannulated, in the same animal, at both ends using also glass cannulae linked with glass syringes. In both groups, the bile duct was ligated to prevent the release of bile salts into the intestinal lumen.

In order to remove all the intestinal contents, each segment was gently washed with perfusion solution free of drug. After complete removal of the intestinal contents, syringes with three-way stopcock valve were coupled to the cannulae and the perfusion solution containing digoxin was introduced into the segment: 2 ml for duodenum, 4 ml for jejunum and ileum, and 10 ml for complete small intestine.

150 µl of the perfusion solution were collected every 5 min for 30 min, alternating the collection end, without replacement. The sample withdrawn volume was, later on, taken into account for water reabsorption estimation.

At the end of the experiment the animals were euthanized by cervical dislocation, the samples were centrifuged at 10000 rpm for 5 min and quantified by HPLC. In addition, the remaining perfusion solution in the intestine was collected to evaluate the degree of water absorption presented by the animal. Water reabsorption was characterized as an apparent order zero process and the water reabsorption constant (k_0) was calculated as described in Eq. (1).

$$K_0 = \frac{V_0 - V_{end}}{t_{end}} \quad (1)$$

where V_0 is the volume of perfusion solution introduced into the intestinal segment at the beginning of the experiment, V_{end} is the volume of perfusion solution measured at the end of the experiment, t_{end} is the total time of experiment (30 min). k_0 value was used to estimate the remaining water volume at each time point (V_t).

Thus, the experimental concentration (C_e) was corrected at each time point using the Eq. (2):

$$C_t = C_e \times \frac{V_t}{V_0} \quad (2)$$

where C_t represents the drug concentration in the absence of water reabsorption at time t, and C_e represents the correct experimental value. C_t values were used to calculate the real absorption rate coefficients (Tugcu-Demiroz et al., 2014).

The absorption rate coefficient (k_a) was determined by nonlinear regression analysis of the remaining concentrations in lumen of segment intestinal (C_t) versus time (Eq. (3)).

$$C_t = C_0 \times e^{-ka.t} \quad (3)$$

From the k_a value is possible to calculate the effective permeability (P_{eff}) using Eq. (4):

$$P_{eff} = \frac{k_a \times r}{2} \quad (4)$$

where r is the radius of the intestinal segment. r was calculated considering the intestinal segment as a cylinder (Volume = $\pi r^2 L$). Estimation was done using the perfusion volume for each intestinal segment.

2.5. Kinetic parameter estimation

A kinetic modelling approach to estimate permeability transport components was applied to the data. The kinetic model was fitted to the whole dataset simultaneously (duodenum, jejunum, ileum and ileum in presence of sodium azide), by keeping in common the shared parameters across segments/conditions and estimating separately the non-shared ones.

This procedure is based in the following assumptions:

a) Same passive diffusion component on the apparent permeability along the complete small intestine, as the experiments were performed at the same luminal pH value.
b) Same K_m value as index of the affinity for the transporter in all intestinal segments.
c) Maximal efflux velocity (V_m) was modelled as a function of Pgp expression level. The procedure is described in Gonzaléz-Álvarez et al. (2007): a baseline value was taken to be the expression level in ileum (V_mI). The value of maximal velocity in duodenum (V_mD) and jejunum (V_mJ) was calculated from the baseline value and a correction factor (Ef), based on mRNA-MDR1 expression reported for Wistar rats (Takara et al., 2003). According to Takara et al. (2003), the expression level in the duodenum is 3.25-fold lower than ileum and 1.44-fold lower in jejunum versus ileum. Then, Ef in duodenum was 1/3.25 and Ef in jejunum was 1/1.44.

The kinetic model is based on four differential equations, one per segment/condition as the next one:

Segments

$$\frac{dC_i}{dt} = -\left(\frac{2}{r_x}P_{diff}\right)C_i + \frac{2}{r_x}\frac{V_{mx} \times C_i}{K_m + C_i} \quad (5)$$

Ileum in presence of sodium azide

$$\frac{dC_{ilaz}}{dt} = -\left(\frac{2}{r}P_{diff}\right)C_{ilaz} \quad (6)$$

where C_i represents the digoxin concentration, P_{diff} the passive diffusion permeability, and K_m is the Michaelis-Menten constant. r represents the effective radius and accounts for the volume/surface ratio. The subscript x refers to segment in question: V_{mD} for duodenum, V_{mJ} for jejunum, and V_{mI} for ileum.

The maximal velocity was estimated to be the product of the baseline value (in ileum) and the expression factor (Ef) for the segment according to Eq. (7) and (8).

$$V_{mD} = Ef_D \times V_{mI} \quad (7)$$

$$V_{mJ} = Ef_J \times V_{mI} \quad (8)$$

Curve fitting procedures were performed in Berkeley-Madonna 8.3.23.0. (apparent permeability values at each concentration/condition) and in Phoenix-Winnonlin 8.0.0.3176. The winnonlin ASCII code is provided in the Table A1.

2.6. Chromatographic conditions

Digoxin was quantified using HPLC system (Waters Alliance® e2695, Milford, MA, USA) with UV detector at 218 nm (Waters 2487, Milford, MA, USA). Using as mobile phase a mixture of 72:28 (v/v) water:acetonitrile and column Nova Pak® Waters (C18 3.9 × 150 mm; 4.0 μm). Temperature was set at 25 °C. Injection volume was 50 μl and flow of 1 ml/minute. The method was previously validated with adequate linearity, precision, accuracy ($R > 0.99$ and coefficient of variation < 5%) and LLOQ of 0.33 μg/ml.

2.7. Statistical analysis

Permeability values are expressed as mean ± standard deviation (SD). Statistical analysis was performed using Graphpad Prism version 5.1 (Graphpad Software, INC., La Jolla, CA, USA). Analysis of variance (ANOVA) and Tukey tests were used for statistics. A value of $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Permeability studies

Cultures of suitable epithelial cells or animal tissues and *in situ* intestinal perfusion in rats are well characterized experimental methods to evaluate intestinal drug permeability in preclinical studies (Lennernäs, 2014, Hens et al., 2016). These methods are accepted by some regulatory agencies to BCS drug classification with regulatory purposes (EMA, 2010, FDA, 2015, WHO, 2015).

Among the epithelial cells, Caco-2 cells deserve attention. Caco-2 cells are derived from human colon adenocarcinoma and were first isolated by Fogh et al. (Fogh and Trempe, 1975). Initially they were used in the investigation of tumor mechanisms of cancer cells and studies on anti-neoplastic therapy (Pinto et al., 1983).

Since the 1980s, Caco-2 cells have become a promising tool for permeability studies because they presente many characteristics of intestinal absorption cells, such as: microvillus, hydrolysis enzymes, ion, peptide and sugar transporters. In addition, to performing both passive and some types of active drug transport (Pinto et al., 1983, Hilgers et al., 1990, Balimane et al., 2000, Souza et al., 2009, Gonçalves et al., 2012).

Despite these characteristics, permeability studies using Caco-2 cells present some disadvantages, such as the long period to obtain viable cells for the tests (usually 21 days) and the significant difference between the expression level of transporters in Caco-2 cells and human intestine. In addition, some experimental problems related to the unstirred water layer, the adherence of highly lipophilic drugs in polycarbonate filters, the pH of donor/receptor media and use of co-solvents, can change the results and make difficult the interlaboratory reproducibility (Balimane et al., 2000, Souza et al., 2007, Gonçalves et al., 2012).

The use of intestinal tissues is also very common. Two techniques for the evaluation of permeability were developed from animal tissues.

Everted intestinal assay utilizes portions of animals intestine, generally rats, in everted form, which are immersed in a buffer solution containing the drug, in order to evaluate the transport from the mucosal to the serosal side (Wilson and Wiseman, 1954, Balimane et al., 2000, Souza et al., 2007).

The second technique uses of intestinal segments in bicompartmental apparatus, usually, Ussing chambers (horizontal) or Franz cells (vertical). The mucosal side faces the donor compartment and the serosal portion faces the receiver one. Absorption is based on the appearance of the drug on the serosal side from disappearance on the mucosal side (Ussing and Zerahn, 1951, Balimane et al., 2000, Souza et al., 2007).

Although these techniques control many of the variables found in the absorption process, they suffer with the main disadvantage of *in vitro* studies: lack of innervation and blood supply. The drug must diffuse not only through the membrane barrier but also through the muscle, connective tissue, fatty tissue, and other structures of intestinal wall. So, as a result, absorption rates measured by these *in vitro* techniques can be slow and accumulation of drug in the “membrane” can be extremely large. In addition, *in vitro* preparations show rapid loss of cell

viability due to morphological changes (Doluisio et al., 1969, Balimane et al., 2000).

In situ intestinal perfusion in rats is performed with several methods but essentially in all of them drug solutions are introduced in the intestinal segment of interest with the animal under anestesia. Absorption is evaluated from the disappearance of the drug from the intestinal lumen. In addition to evaluate the permeability, this technique allows the analysis of the kinetic mechanism of drug absorption (Doluisio et al., 1969, Balimane et al., 2000, Tugcu-Demiroz et al., 2014, Lozoya-Agullo et al., 2016).

Blood supply and innervation are maintained, thus the technique is able to mimic the *in vivo* conditions. However, some limitations are observed as the fact that the absorption is correlated only with the disappearance of the compound from the intestinal lumen, thus disregarding any pre-systemic or luminal metabolism (Doluisio et al., 1969, Balimane et al., 2000).

In recent years, *in situ* intestinal perfusion in rats with blood sampling has gained focus on permeability studies of substances. Cannulation of the mesenteric vein allows determining the appearance of compounds in the blood and is of great use, especially, when evaluating the absorption of compounds submitted to intestinal metabolism. In addition, rats expres intestinal transporters and P450 enzymes, then, *in situ* intestinal perfusion in rats has been used to study the intestinal absorption of drugs that are affected by intestinal metabolism and efflux transporters. For some compounds, it is possible to monitor the appearance of metabolites, originating from intracellular metabolism, in the perfusion media. These metabolites can reach the intestinal lumen by active or passive transport processes and serve as a measure of the metabolism (Stappaerts et al., 2015, Dezani et al., 2017).

To compare the prediction of drug absorption by different methods, Lozoya-Agullo et al. (2015a) evaluated the permeability of several compounds using *in situ* intestinal perfusion and Caco-2 cells. Differences in permeability ranges in cell culture compared to *in situ* perfusion models were observed for all drugs. This is a reflection of the difference in the surface area available for transport (in the cellular model there are only villus, while in the animal model there are villus and folds) and in the expression of transporters (Lozoya-Agullo et al., 2015a).

Although human data are considered most reliable in comparison with *in vitro* and *in situ* intestinal perfusion studies, ethical and bureaucratic issues, besides the high cost of studies, should not be ignored. Ethical issues are also involved in the use of animals and experimental protocols must be approved by ethics committees. However, *in situ* intestinal perfusion is a simple, inexpensive and fast method, and presents a good correlation with the permeability and absorbed fraction data in humans when compared to other *in vitro* studies, mainly for drugs absorbed by passive diffusion (main route for drug absorption) (Balimane et al., 2000, Cao et al., 2006, Volpe, 2010, Lozoya-Agullo et al., 2015b). Futhermore, this method can be used to evaluate the carrier-mediated mechanisms on permeation process (Cao et al., 2006, Dezani et al., 2017). A single animal produces suitable experimental data for complete kinetic analysis, being four animals the minimum number for obtaining relevant, reliable and safe data (Doluisio et al., 1969).

3.2. Variations of in situ intestinal perfusion

In order to improve and become closer to the reality of the human organism, several variations were developed for *in situ* intestinal perfusion technique: single-pass intestinal perfusion (Amidon et al., 1981), recirculating perfusion (Van Rees et al., 1974), oscillating perfusion (Schurgers and De Blaey, 1984) and closed-loop method (Doluisio et al., 1969). Among them, single-pass intestinal perfusion and closed-loop or Doluisio method have more widespread application.

In single-pass intestinal perfusion (SPIP), the drug solution is perfused, at a constant flow through a cannula, into 10–15 cm of intestinal segment. Samples are collected in the distal region, in which the intestine was cannulated, at predetermined times (Amidon et al., 1981). Effective permeability (P_{eff}) is calculated from the relationship between the initial and final concentrations of the drug, the flow and the intestinal segment area, according to Eq. (9) (Dahan et al., 2009).

$$P_{eff} = \frac{-Q.\ \ln\left(\frac{C_f}{C_i}\right)}{2\pi rL} \quad (9)$$

where Q is the perfusion flow rate, Cf/Ci is the ratio of the final and the initial concentration of drug that has been adjusted for water absorption. r is the radius of the intestinal segment and L is the length of the perfused intestinal segment.

In the closed-loop method, the intestinal segment is completely isolated with two cannulas at the beginning and at the end of the segment. Drug solution is perfused and samples are collected at predetermined times. The results are highly reproducible and produce absorption rates realistic and comparable to those calculated from blood concentration from oral administration of drug to humans and animals. The disappearance of drug from the intestinal lumen in the time frame of the experiments (30–60 min) follows an apparent first-order kinetic process. Moreover, the experimental technique is simple, utilizes readily available laboratory equipment and small volume of perfusion solution, when compared to SPIP, an advantage when only small amounts of compound are available (Doluisio et al., 1969).

In SPIP method permeability is estimated from the ratio of inlet and outlet concentration in the intestinal segment after reaching steady-state while in Closed loop method used in this study we are not under steady-state conditions and we are estimating permeability from the disappearance rate. We have performed a extensive research in order to compare both perfusion methods and calculation approaches and we have demonstrated a very good correlation between both experimental approach, not only for digoxin but also for a extensive dataset of compounds of different physicochemical characteristics and in diferente rat intestinal segments. Both techniques perform similarly in terms of the obtained Peff values which are comparable in range and in variability.

Lozoya-Agullo et al. (2015b) evaluated the *in situ* intestinal perfusion of 15 model drugs with different permeability characteristics (low, moderate, and high, as well as passively and actively absorbed), in both SPIP and Doluisio (closed-loop) methods. An excellent correlation (coefficient of determination (r2) of 0.93) was obtained for the P_{eff} values using the two techniques. Consequently, the absorbed fraction predicted from the permeability values showed a good correlation with human data. In addition, for 14 of the 15 drugs evaluated, the permeability class according to BCS presented, for both methods, was similar to the data reported in the literature (Lozoya-Agullo et al., 2015b).

The comparison of SPIP and Doluisio method has also been performed using intestinal segments such as upper jejunum, mid-small intestine, ileum and colon. Good correlation between the SPIP and Doluisio approaches was obtained for jejunum ($r^2 = 0.95$) and mid-small intestine ($r^2 = 0.9$), while for ileum ($r^2 = 0.85$) and colon ($r^2 = 0.81$), lower values were reported (Lozoya-Agullo et al, 2015b, Lozoya-Agullo et al., 2016, Lozoya-Agullo et al., 2017).

Permeability is dependent on the intestinal region (Dahan et al., 2009), due to this fact, despite the good correlations between SPIP and Doluisio, the second method makes it possible to use any length of intestinal segment (SPIP can only use 10–15 cm), and thus to evaluate the action of transporters better.

It is known that the expression of transporters changes throughout the gastrointestinal tract. For example, two efflux transporters of the small intestine stand out: multidrug-resistance protein 2 (MRP2) and P-glycoprotein (Pgp). Both are located in apical region of the enterocytes, however, MRP2 shows greater expression in proximal region of the small intestine while Pgp has increased expression along the gastrointestinal tract, presenting a higher concentration in the ileum (Yao and Chiou, 2006, Tamaki et al., 2011, Dahan et al., 2012).

Table 1
Absorption rate coefficients and effective permeability values obtained by *in situ* perfusion method in rat for digoxin (mean ± SD).

Concentration	ka (min^{-1})	P_{eff} (cm/s)
1.0 µg/ml	0.016 ± 0.008	$2.36 \times 10^{-5} \pm 1.25 \times 10^{-5}$
6.0 µ g/ml	0.031 ± 0.005	$4.57 \times 10^{-5} \pm 7.96 \times 10^{-6}$
1.0 µ g/ml with sodium azide	0.040 ± 0.020	$5.58 \times 10^{-5} \pm 2.61 \times 10^{-5}$
Intestinal segment		
Duodenum	0.013 ± 0.003	$3.61 \times 10^{-5} \pm 5.53 \times 10^{-6}$
Jejunum	0.018 ± 0.006	$2.69 \times 10^{-5} \pm 7.37 \times 10^{-6}$
Ileum	0.009 ± 0.005	$1.35 \times 10^{-5} \pm 7.45 \times 10^{-6}$

Intestinal perfusion in rats using both closed-loop and single-pass methods provided similar effective permeability values, demonstrating the robustness of the techniques and the possibility of combining data from different laboratories (Lozoya-Agullo et al., 2015b). However, due to the technical simplicity, greater speed and good correlation, the closed-loop method was chosen to carry out the study of digoxin.

3.3. A digoxin study

Permeability of digoxin was evaluated throughout the complete small intestine and in the intestinal segments duodenum, jejunum and ileum.

Absorption rate coefficients (k_a) and effective permeability (P_{eff}) values obtained for digoxin using the *in situ* intestinal perfusion technique in rats are shown in Table 1.

Fig. 1 shows comparative graphs of P_{eff} values obtained for digoxin at the different concentrations (1.0 µg/ml with and without sodium azide and 6.0 µg/ml) used in the study. Sodium azide is a non-specific metabolic inhibitor that produces a decrease in ATP levels due to interference in the transport of electrons in the mitochondrial matrix. Therefore, as the transport system in the rat model was ATP-dependent, sodium azide is able to inhibit the digoxin transport by any type of carrier (uptake and efflux), resulting in the passive permeability of the drug (Valenzuela et al., 2001, Fernandez-Teruel et al., 2005). The passive permeability of digoxin in presence of sodium azide was about 2-fold higher than effective permeability with both components passive plus secretion carrier-mediated.

Fig. 2 shows comparative graphs of P_{eff} values obtained for digoxin 1.0 µg/ml in the different intestinal segments. No statistically significant differences were observed between duodenum and jejunum, however statistically significant differences were observed between jejunum and ileum ($p < 0.05$) and between duodenum and ileum ($p < 0.01$).

In order to estimate the kinetic parameters, in addition to the P_{eff} values obtained for clinical doses of digoxin, P_{eff} data of digoxin at 10.0 µM (7.8 µg/mL) were reported from Lozoya-Agullo et al. (2016). According to Lozoya-Agullo et al. (2016), digoxin at 10.0 µM showed P_{eff} ($\pm$ SD) of 3.87×10^{-5} ($\pm 1.01 \times 10^{-5}$), 2.93×10^{-5} ($\pm 1.19 \times 10^{-6}$), 2.18×10^{-5} ($\pm 8.14 \times 10^{-6}$), in the intestinal segments duodenum, jejunum and ileum, respectively. Table 2 shows the estimated parameters from the kinetic model described in Eq. (5)–(8).

As it can be seen in Table 2 the estimated passive diffusional component P_{diff} is closer to the experimental value obtained in presence of sodium azide when all the carrier-mediated processes are inhibited. On the other hand, the estimated K_m value is higher than the experimental concentration used in this study and similar to the concentration used by Valizadeh et al. (2012). This fact confirms that the efflux component is not saturated at the clinically relevant concentrations.

A previous attempt to characterize the permeability of digoxin in intestinal segments of rats was performed by Stephens et al. (2001), using excised segments. A P-gp mediated efflux was also demonstrated, which was higher in ileum in accordance with the higher expression levels in this segment, and estimated K_m values ranged from 51 to 81 µM (Stephens et al., 2001). This divergence between K_m values (in this work and in Stephens et al. (2001)) can be due to the differences in experimental conditions (excised segment versus *in situ* perfusion, and different perfusion buffer). On the other hand the V_m values reported in jejunum and ileum (4.7 and 9.3 nmol/cm^2/h) are closer to our range observed for Vm (1.48 and 2.13 nmol/cm^2/h), which is a reasonable difference considering all the changes in experimental variables.

As shown in Fig. 3, the kinetic model presents a good predictive performance considering the simplicity of the assumptions, the diffusional component is corrected based on the surface/volume ratio by using a different radius value (0.25 cm for duodenum; 0.18 cm for jejunum and 0.17 for ileum) and the expression levels reported by Takara et al. (2003) to estimate the V_m in each segment.

Digoxin is rapidly absorbed, with peak serum concentrations occurring within 1.5–6 h after administration of the oral dose. Tablets have a bioavailability of 70–80%, while gel capsules and elixirs approach 100%. The bioavailability of digoxin does not appear to change significantly with advancing age, although the rate of absorption is faster in younger patients (Borron et al., 1997, Hanratty et al., 2000).

From effective permeability values for digoxin (lower than metoprolol), the fraction absorbed (F_a) in humans was predicted by Lozoya-Agullo et al. (2015b). Digoxin had F_a predicted in the range of 72–81%, i.e., values closer to the tablets bioavailability and to the reported in the literature (81%) by Varma et al. (2012). Regarding to capsules and elixirs, the increased bioavailability can be related to the presence of excipients able to inhibit the efflux transporter thus leading to a higher permeability value and to a complete fraction absorbed.

Digoxin has a molecular weight of 780.9 g/mol, LogP of 1.46 and 1.95, CLogP of 1.27 and apparent linear absorption, therefore it is considered a lipophilic drug absorbed mainly by passive transcellular route (Kasim et al., 2004). There are reports suggesting the involvement of the OATP-mediated mechanism in the uptake of digoxin. While it is well known that digoxin is a substrate of the P-glycoprotein (Pgp), efflux transporter located on the apical surface of enterocytes and responsible for pumping drugs back into the intestinal lumen (Gandia

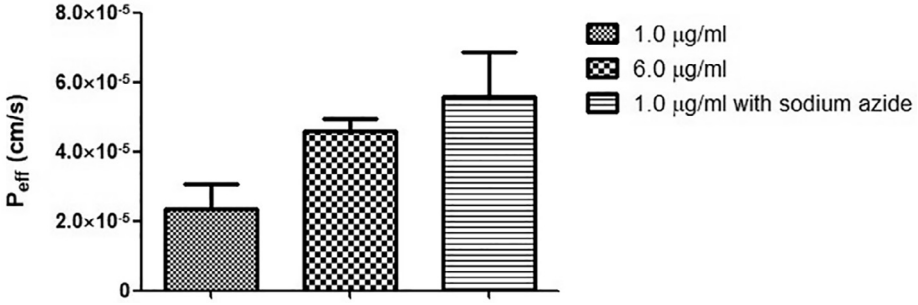

Fig. 1. P_{eff} values obtained by *in situ* perfusion method in rats (n = 4–6) for digoxin 1.0 µg/ml, 6.0 µg/ml and 1.0 µg/ml with sodium azide (mean ± SD). No statistically significant differences were observed ($p > 0.05$).

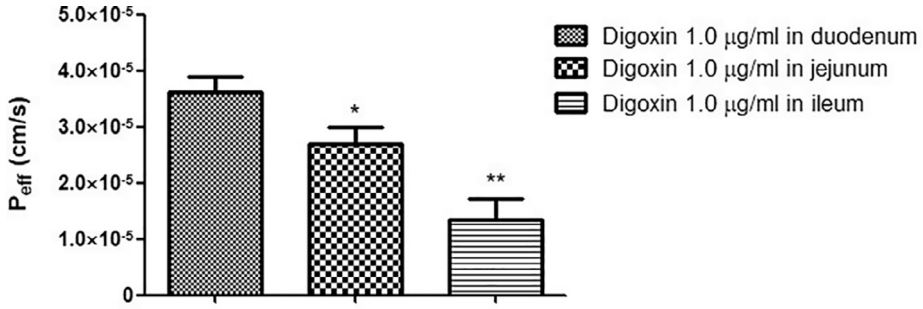

Fig. 2. P_{eff} values obtained by *in situ* perfusion method in rats (n = 4–6) for digoxin 1.0 μg/ml in the intestinal segments duodenum, jejunum and ileum (mean ± SD). * Significant difference between jejunum and ileum ($p < 0.05$). ** Significant difference between duodenum and ileum ($p < 0.01$).

Table 2
Parameters obtained by curve fitting of Eqs. (5)–(8) to the experimental data (concentrations versus time profiles in the three studied segments).

Parameter	Value
P_{diff}	5.97×10^{-5} cm/s
K_m	10.73 μg/ml
V_mD	1.77×10^{-3} μg/ml/s
V_mJ	4.00×10^{-3} μg/ml/s
V_mI	5.77×10^{-3} μg/ml/s
r	0.968
SSR	0.206

r = coefficient of correlation and SSR = residual sum of squares.

et al., 2004, Yao and Chiou, 2006, Suzuki et al., 2014, Nielsen et al., 2016, Oda and Murakami, 2017).

P-glycoprotein (Pgp) is widely distributed in the body and is found in epithelial and endothelial tissues, such as the intestinal mucosa and blood-brain barrier, and in organs, such as the liver and kidney. Increasing its importance in drug interactions involving digoxin and in the secretion of the drug along the tissues in the organism (Thiebaut et al., 1989, Fenner et al., 2009, Cook et al., 2010, Suzuki et al., 2014).

Regarding drug interactions, Fenner et al. (2009) and Cook et al. (2010) evaluated the clinical relevance of drug-drug interactions mediated through Pgp using digoxin as model drug. These authors identified that many coadministered drugs (co-meds) with digoxin are cardiovascular agents, which caused a clinically significant drug interaction (25% change in AUC ratio). The presence of cardiovascular agents may alter cardiac output and/or renal activity, acting alone or being additional components to increase digoxin exposure along with Pgp interaction (Fenner et al., 2009, Cook et al., 2010).

Analysis of 123 study reports examining digoxin pharmacokinetics in the presence and absence of co-meds (substrates or inhibitors of Pgp) showed only small changes in pharmacokinetic parameters. In addition, inhibition of Pgp is more pronounced in the intestine, since systemic

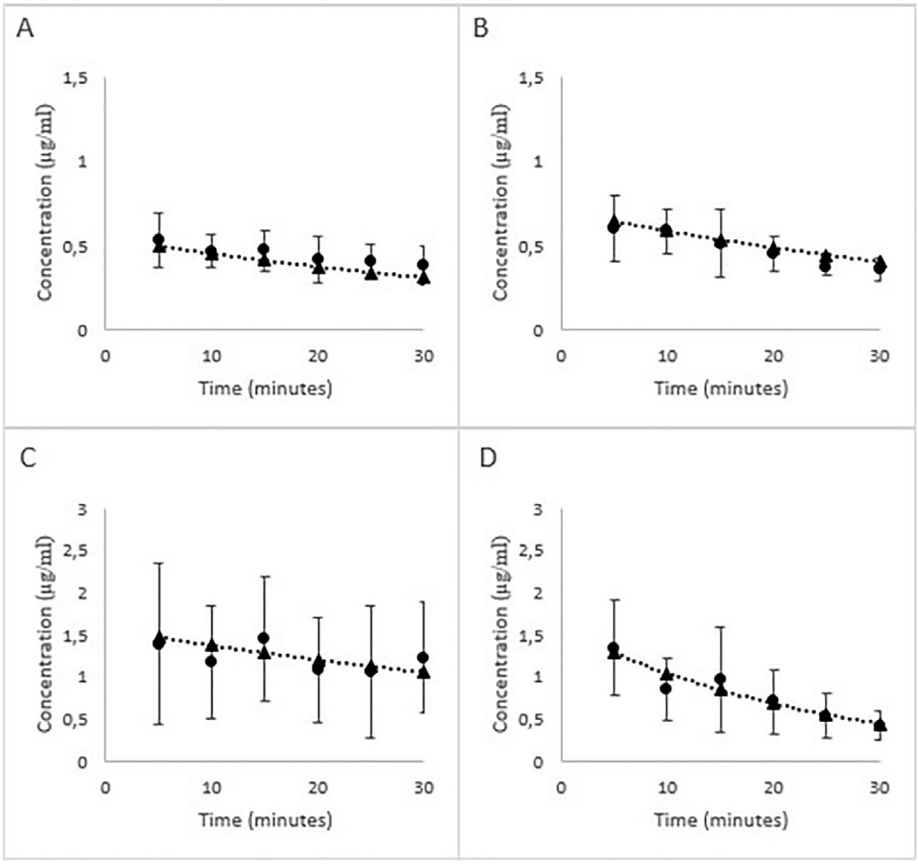

Fig. 3. Mean experimental concentrations (●) obtained by *in situ* perfusion method in rats (n = 4–6) for digoxin and the model (Eq (5)-(8) predicted ones (▲) in: (A) duodenum, (B) jejunum, (C) ileum and (D) ileum in presence of sodium azide.

inhibition would cause an increase in exposure < 25% (Fenner et al., 2009).

Digoxin administered as tablets is approximately 70% absorbed. If Pgp only limits the absorption of digoxin, then a maximum increase of 42% in exposure (AUC) would be expected when coadministered with a potent inhibitor acting only in the intestine. In contrast, digoxin administered as soft gelatin capsule is almost completely absorbed, not expecting changes in exposure when coadministered with a similar inhibitor, thus being less susceptible to clinically important interactions. Less drug-drug interactions were observed after intravenous administration of digoxin, suggesting that interactions resulting in digoxin toxicities may be a greater concern with tablets rather than capsules (complete absorption in the upper gastrointestinal tract) (Fenner et al. 2009, Cook et al., 2010).

In the intestine, inhibition of the Pgp by coadministration of drugs can increase the absorption of its drug substrates. Due to its narrow therapeutic index, interactions with digoxin are particularly important because any inhibition of the Pgp action can lead to potentially toxic increases in systemic circulation. Pgp is also expressed at high levels in the liver, kidneys and blood-brain barrier, then, interactions mediated by the competitive inhibition of this efflux transporter can also have significant effects on tissue excretion and distribution, especially in the penetration of drugs into the brain (Collett et al., 2005, Fenner et al., 2009).

Intestinal secretion of drugs is an important clearance mechanism that also affects the oral bioavailability of many drugs. Intestinal secretory capacity does not only affect absorption kinetics but also contributes to total clearance of cardiac glycosides (Hunter and Hirst, 1997).

Mayer et al. (1996) showed this by evaluating the excretion of digoxin and its distribution along the tissues in wild-type mices and in mices with a disrupted mdr1a gene (mdr1a (−/−) mice), i.e., mices without expression of Pgp (Mayer et al., 1996).

Intestinal Pgp contributes to a substantial excretion of digoxin through the intestinal epithelium, not only being important for the elimination of the drug from the systemic circulation, but also for a decrease in the intestinal re-uptake of digoxin after biliary excretion, modulating its absorption along the intestine (Mayer et al., 1996).

In the brain of wild-type mices, digoxin levels remained very low, while in mdr1a (−/−) mices these levels gradually increased over 3 days, resulting in approximately 200-fold difference from wild-type mices in the same period. The prolonged accumulation of digoxin in brains of mdr1a (−/−) mices reinforces the pharmacological importance of Pgp in the blood-brain barrier, making doctors and scientists aware of a possible increased risk of toxicity in patients exposed to coadministration of substrates or inhibitors of Pgp with digoxin (Mayer et al., 1996).

Many studies performed to provisionally classify drugs according to the BCS, only evaluate solubility data and LogP (experimental) or CLogP (predicted) values, which frequently can overestimate or infra-estimate experimental permeability if there are carrier-mediated processes, as the lipophilicity is a good predictor only of the passive diffusional component. For example, digoxin is often provisionally classified as BCS class I or II (Kasim et al., 2004, Lindenberg et al., 2004, Wu and Benet, 2005, Butler and Dressman, 2010).

Other studies using different techniques have demonstrated the divergence regarding the permeability of digoxin.

Varma and Panchagnula (2005) evaluated the permeability for several compounds, among them, digoxin, using *in situ* single-pass perfusion method in rat. Intestinal perfusion of digoxin at 20 μM (15.6 μg/ml) was performed along 12–15 cm of ileum, and showed P_{eff} equal to 0.04×10^{-4} cm/s. This value indicates low permeability because only drugs with P_{eff} higher than 0.2×10^{-4} cm/s are completely absorbed (Varma et al., 2004, Varma and Panchagnula, 2005).

Valizadeh et al. (2012) evaluated the permeability of digoxin using *in situ* permeation studies in rats in 10 cm of jejunum and four different concentrations of digoxin. Effective permeability (cm/s) of digoxin at concentrations of 5, 10, 15 and 20 μM (3.9, 7.8, 11.7 and 15.6 μg/ml) were 3.0×10^{-5}, 2.9×10^{-5}, 3.2×10^{-5} and 2.4×10^{-5}, respectively. Digoxin was classified as BCS class IV, because it presented, in addition to $P_{eff} < 5.09 \times 10^{-5}$ cm/s (on the basis of the relationship between human and rat intestinal permeability, P_{eff} values in rats > 5.09×10^{-5} cm/s correspond to Fa > 85%), a dose number (Do) greater than 1 (Zakeri-Milani et al., 2009, Valizadeh et al., 2012).

In a third study, permeability values of digoxin were determined using the 2/4/A1 cell line (2/4/A1 is rat fetal intestinal epithelial cell and has a paracellular permeability comparable with human small intestinal epithelium, showing a better correlation with human effective permeability). The cutoff value for high permeability compounds is 55×10^{-6} cm/s in this cell line. So, digoxin (range from 0.1 mM (78.0 μg/ml) to 1.0 mM (780.0 μg/ml)) with P_{app} equal to 56, 1×10^{-6} cm/s was considered highly permeable (Zaki et al., 2010).

Transport studies across the rat ileum using Ussing chambers were performed and it was observed that only about 3–4% of perfused digoxin (10 μM–7.8 μg/ml) was absorbed. Demonstrating P_{app} between 8 and 10×10^{-6} cm/s (Oga et al., 2013). While in an intestinal absorption study in rats using *in situ* closed-loop perfusion method, digoxin at concentrations of 10 (7.8 μg/ml) and 100 μM (78.0 μg/ml), presented an estimated absorption of 27 and 53%, respectively (Suzuki et al., 2014).

Due to this divergence of values and to the fact that most studies have been performed at concentrations higher than clinical concentrations (i.e dose/administered volume assuming complete dissolution) not ensuring the saturation of efflux transporters, and consequently overestimating permeability values, it was decided to perform the permeability studies of digoxin, in clinical doses/concentration, using *in situ* closed-loop perfusion method in rats.

The results obtained (Table 1) for P_{eff} of digoxin were analyzed by comparison with the P_{eff} values obtained for metoprolol (high permeability standard). Drugs that have permeability values below the P_{eff} value of metoprolol are considered poorly permeable.

Ruiz-Picazo et al. (2017) using *in situ* perfusion in rats study based in Doluisio method found that metoprolol shows P_{eff} of 6.24×10^{-5} cm/s, 6.85×10^{-5} cm/s and 9.05×10^{-5} cm/s for complete small intestine, jejunum and ileum, respectively (Ruiz-Picazo et al., 2017).

Beyond to the comparison with metoprolol, the P_{eff} values obtained for digoxin were evaluated according to the guidelines established by Zakeri-Milani et al. (2009): a drug is highly permeable when exhibits P_{eff} (rat) > 5.09×10^{-5} cm/s, and of low permeability when P_{eff} (rat) < 4.2×10^{-5} cm/s. Drugs with effective permeability between 4.2×10^{-5} and 5.09×10^{-5} cm/s were considered as borderline (Zakeri-Milani et al., 2009).

Clinical doses of 0.25 mg (1.0 μg/ml) and 1.5 mg (6.0 μg/ml) showed P_{eff} below the P_{eff} value for metoprolol (6.4×10^{-5} cm/s) as well as below the limits established by Zakeri-Milani et al. (2009). In addition, both concentrations had P_{eff} values below the passive permeability of digoxin (digoxin with sodium azide) indicating that administration of these two doses did not saturate the efflux transporter.

Regarding the intestinal segments, a greater effective permeability was observed in the duodenum and jejunum (no statistically significant differences were observed between these two segments $p > 0.05$) when compared to ileum (Table 1 and Fig. 2).

Permeability is dependent on the intestinal region. In addition to the heterogeneous expression of efflux transporters (e.g. MRP2 and Pgp), the regional permeability is determined by the physicochemical properties of the drug and the function of the membrane and the pH of intestinal fluids. The intestinal segments differ in many aspects, being the total lipid surface area, tight junctional resistance and area, enzymatic activities, amount and capacity of transporters, water flow, unstirred water layer and capillary blood flow, the main factors affecting permeability (Fagerholm et al., 1997, Ungell et al., 1998). However, the

segmental absorption of digoxin points out the differential contribution of the efflux component associated with the different expression level. Distribution of P-glycoprotein is not homogeneous along the intestine and there is a progressive increase in the function/expression of Pgp from duodenum to ileum (Yao and Chiou, 2006, Nishimura et al., 2008). Incorporating in the mathematical model the reported expression levels has permitted a good prediction of the concentrations versus time digoxin intestinal profiles.

Such in the complete small intestine, the P_{eff} values obtained for digoxin in the intestinal segments were lower than P_{eff} values for metoprolol and the limit imposed by Zakeri-Milani et al. (2009) for highly permeable drugs.

Therefore, the results obtained using *in situ* perfusion technique based on Doluisio method, in these experimental conditions, along with the paremeter estimation based in a kinetic model involving passive and efflux mechanism suggest the low permeability of digoxin at the clinical concentrations, with a decrease in the drug permeation along the small intestine.

4. Conclusion

Among the techniques recommended by regulatory agencies for the classification of drugs according to BCS, intestinal perfusion in rats has been widely used due to simplicity, low cost and throughput when compared to *in vivo* studies in humans. In comparison with Caco-2 cells method, even if the cell culture could provide a higher throughput for screening, the rat presents more similar expression of transporters and the advantage of segmental comparisons. In summary, it shows good correlation with human data, due to physiological and morphological similarities between the two species.

The application of the *in situ* perfusion technique in rats allowed the evaluation of the effective permeability of digoxin, in clinically relevant concentrations (i.e dose/250 ml), suggesting the low permeability of this drug, due to lower P_{eff} values when compared with metoprolol and to the cutoff value proposed by Zakeri-Milani et al. (2009). The estimated K_m value demonstrated that at clinical concentrations the efflux process is not saturated and then it could be inhibited by other drugs, excipients or food components leading to the already reported clinical drug-drug and drug-food interations. The present data confirms from a mechanistic point of view these interactions.

It should be emphasized that the techniques used to classify the permeability of drugs according to the guidelines of the BCS must be standardized. It is essential to know the variables and possible modifications in the experimental procedure, in order to apply the most suitable model to optimize the results, generating data as close as possible to the reality of the human organism.

Acknowledgments

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil, fellowship Edital n° 19/2016) for financial support to the stage of Tamires Guedes Caldeira in Universidad Miguel Hernandez. Partial support from “MODELOS *IN VITRO* DE EVALUACION BIOFARMACEUTICA” SAF2016-78756 (AEI/FEDER, EU) funded by Agencia Estatal Investigación and European Union, through FEDER (Fondo Europeo de Desarrollo Regional).

Appendix

See

Table A1
Winnonlin ASCII code.

Model 1

```
remark
    ********************************************************
remark Developer: Marival Bermejo
remark Digoxin absorption model in rat intestinal segments at
    remark different initial concentrations and in presence of sodium azide
remark Model Date: 07-26-2017
remark
    ********************************************************
remark – define model-specific commands
COMMANDS
NFUNCTIONS 4
NDERIVATIVES 4
NPARAMETERS 3
PNAMES 'Pdiff','km','vm'
Ncons 3
END
remark – define temporary variables
TEMPORARY
Pdiff = P(1)
km = P(2)
vm = P(3)
A = con(1)
e1 = con(2)
e2 = con(3)
remark A = Lduo/lileon 0.222
remark e1 = 1/3.25 and e2 = 1/1.44 from Takara et al.
t = x
END
remark – define differential equations starting values
START
Z(1) = 0.55
Z(2) = 0.7082
```

Table A1 (*continued*)

Model 1
Z(3) = 1.58
Z(4) = 1.59
END
remark - define differential equations
rema Z(1) duodenum Z(2) jejunum
rema Z(3) ileum Z(4) ileum + azida
DIFF
DZ(1) = −A * (2 * (Pdiff/0.25) * Z(1)) + (e1 * Vm * Z(1)/(km + Z(1)))
DZ(2) = −(2 * (Pdiff/0.18) * Z(2)) + (e2 * vm * Z(2)/(km + Z(2)))
DZ(3) = −(2 * (Pdiff/0.17) * Z(3)) + (vm * Z(3)/(km + Z(3)))
DZ(4) = −2 * (Pdiff/0.17) * Z(4)
END
remark – define algebraic functions
FUNCTION 1
F = Z(1)
END
FUNCTION 2
F = Z(2)
END
FUNCTION 3
F = Z(3)
END
FUNCTION 4
F = Z(4)
END
EOM

References

Amidon, G.E., Ho, N.F.H., French, A.B., Higuchi, W.I., 1981. Predicted absorption rates with simultaneous bulk fluid flow in the intestinal tract. J. Theor. Biol. 89, 195–210. https://doi.org/10.1016/0022-5193(81)90306-4.

Amidon, G.L., Lennernäs, H., Shah, V.P., Crison, J.R., 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. https://doi.org/10.1023/A:1016212804288.

Balimane, P.V., Chong, S., Morrison, R.A., 2000. Current methodologies used for evaluation of intestinal permeability and absorption. J. Pharmacol. Toxicol. Methods 44, 301–312. https://doi.org/10.1016/S1056-8719(00)00113-1.

Borron, S.W., Bismuth, C., MuszynskI, J., 1997. Advances in the management of digoxin toxicity in the older patient. Drugs Aging 10, 18–33. https://doi.org/10.2165/00002512-199710010-00003.

Butler, J.M., Dressman, J.B., 2010. The developability classification system: application of biopharmaceutics concepts to formulation development. J. Pharm. Sci. 99, 4940–4954. https://doi.org/10.1002/jps.22217.

Cao, X., Gibbs, S.T., Fang, L., Miller, H.A., Landowski, C.P., Shin, H.C., Lennernas, H., Zhong, Y., Amidon, G.L., Yu, L.X., Sun, D., 2006. Why is it challenging to predict intestinal drug absorption and oral bioavailability in human using rat model. Pharm. Res. 23, 1675–1685. https://doi.org/10.1007/s11095-006-9041-2.

Collett, A., Tanianis-Hughes, J., Carlson, G.L., Harwood, M.D., Warhurst, G., 2005. Comparison of P-glycoprotein-mediated drug–digoxin interactions in Caco-2 with human and rodent intestine: Relevance to in vivo prediction. Eur. J. Pharm. Sci. 26, 386–393. https://doi.org/10.1016/j.ejps.2005.07.013.

Cook, J.A., Feng, B., Fenner, K.S., Kempshall, S., Liu, R., Rotter, C., Smith, D.A., Troutman, M.D., Ullah, M., Lee, C.A., 2010. Refining the in vitro and in vivo critical parameters for P-Glycoprotein, [I]/IC50 and [I_2]/IC50, that allow for the exclusion of drug candidates from clinical digoxin interaction studies. Mol. Pharm. 7, 398–411. https://doi.org/10.1021/mp900174z.

Dahan, A., West, B.T., Amidon, G.L., 2009. Segmental-dependent membrane permeability along the intestine following oral drug administration: evaluation of a triple single-pass intestinal perfusion (TSPIP) approach in the rat. Eur. J. Pharm. Sci. 36, 320–329. https://doi.org/10.1016/j.ejps.2008.10.013.

Dahan, A., Lennernäs, H., Amidon, G.L., 2012. The fraction dose absorbed, in humans, and high jejunal human permeability relationship. Mol. Pharm. 9, 1847–1851. https://doi.org/10.1021/mp300140h.

Dezani, T.M., Dezani, A.B., Silva, M.M.C., Serra, C.H.R., 2017. *In situ* intestinal perfusion in rodents: future perspectives for application on absorption studies and classification of drugs. Mini Rev. Med. Chem. 17, 746–757. https://doi.org/10.2174/1389557516666160921145613.

Doluisio, J.T., Billups, N.F., Dittert, L.W., Sugita, E.T., Swintosky, J.V., 1969. Drug absorption I: an *in situ* rat gut technique yielding realistic absorption rates. J. Pharm. Sci. 58, 1196–1200. https://doi.org/10.1002/jps.2600581006.

EMA (2010). Guideline on the investigations of bioequivalence. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/01/WC500070039.pdf (Accessed 01.12.2017).

Fagerholm, U., Lindahl, A., Lennernäs, H., 1997. Regional intestinal permeability in rats of compounds with different physicochemical properties and transport mechanisms. J. Pharm. Pharmacol. 49, 687–1490. https://doi.org/10.1111/j.2042-7158.1997.tb06093.x.

FDA (2015). Waiver of in vivo bioavailability and bioequivalence studies for immediate-release solid oral dosage forms base on a Biopharmaceutics Classification System. http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070246.pdf (Accessed 01.12.17).

Fenner, K.S., Troutman, M.D., Kempshall, S., Cook, J.A., Ware, J.A., Smith, D.A., Lee, C.A., 2009. Drug-Drug interactions mediated through P-Glycoprotein: clinical relevance and In Vitro–In Vivo correlation using digoxin as a probe drug. Clin. Pharmacol. Ther. 85, 173–181. https://doi.org/10.1038/clpt.2008.195.

Fernandez-Teruel, C., González-Álvarez, I., Casabó, V.G., Ruiz-Garcia, A., Bermejo, M., 2005. Kinetic modelling of the intestinal transport of sarafloxacin. Studies *in situ* in rat and *in vitro* in Caco-2 cells. J. Drug Target. 13, 199–212. https://doi.org/10.1080/10611860500087835.

Fogh, J., Trempe, G., 1975. New human tumor cell lines. In: Fogh, J. (Ed.), Human Tumor Cells In Vitro. Plenum, New York, pp. 115–141.

Gandia, P., Lacombe, O., Woodley, J., Houin, G., 2004. The perfused everted intestinal segment of rat. Arzneim-Forsch/Drug Res. 54, 467–473.

Gonçalves, J.E., Fernandes, M.B., Chiann, C., Gai, M.N., Souza, J., Storpirtis, S., 2012. Effect of pH, mucin and bovine serum on rifampicin permeability through Caco-2 cells. Biopharm. Drug Dispos. 33, 316–323. https://doi.org/10.1002/bdd.1802.

Gonzaléz-Álvarez, I., Fernández-Teruel, C., Casabó-Alós, V.G., Garrigues, T.M., Polli, J.E., Ruiz-García, A., Bermejo, M., 2007. *In situ* kinetic modelling of intestinal efflux in rats: functional characterization of segmental diferences and correlation with *in vitro* results. Biopharm. Drug Dispos. 28, 229–239. https://doi.org/10.1002/bdd.548.

Hanratty, C.G., McGlinchey, P., Johnston, G.D., Passmore, A.P., 2000. Differential pharmacokinetics of digoxin in elderly patients. Drugs Aging 17, 353–362. https://doi.org/10.2165/00002512-200017050-00003.

Hens, B., Corsetti, M., Spiller, R., Marciani, L., Vanuytsel, T., Tack, J., Talattof, A., Amidon, G.L., Koziolek, M., Weitschies, W., Wilson, C.G., Bennink, R.J., Brouwers, J., Augustijns, P., 2016. Exploring gastrointestinal variables affecting drug and formulation behavior: methodologies, challenges and opportunities. Int. J. Pharm. 519, 79–97. https://doi.org/10.1016/j.ijpharm.2016.11.063.

Hilgers, A.R., Conradi, R.A., Burton, P.S., 1990. Caco-2 cell monolayers as a model for drug transport across the intestinal mucosa. Pharm. Res. 7, 902–910. https://doi.org/10.1023/A:1015937605100.

Hunter, J., Hirst, B.H., 1997. Intestinal secretion of drugs. The role of P-glycoprotein and related drug efflux systems in limiting oral drug absorption. Adv. Drug Deliv. Rev. 25, 129–157. https://doi.org/10.1016/S0169-409X(97)00497-3.

Kasim, N.A., Whitehouse, M., Ramachandran, C., Bermejo, M., Lennernäs, H., Hussain, A.S., Junginger, H.E., Stavchansky, S.A., Midha, K.K., Shah, V.P., Amidon, G.L., 2004. Molecular properties of who essential drugs and provisional biopharmaceutical classification. Mol. Pharm. 1, 85–96. https://doi.org/10.1021/mp034006h.

Lautenschläger, I., Dombrowsky, H., Frerichs, I., Kuchenbecker, S.C., Bade, C., Schultz, H., Zabel, P., Scholz, J., Weiler, N., Uhlig, S., 2010. A model of the isolated perfused rat small intestine. Am. J. Physiol. Gastrointest. Liver Physiol. 298, 304–313. https://doi.org/10.1152/ajpgi.00313.2009.

Lennernäs, H., 1997. Human jejunal effective permeability and its correlation with preclinical drug absorption models. J. Pharm. Pharmacol. 49, 627–638. https://doi.org/10.1111/j.2042-7158.1997.tb06084.x.

Lennernäs, H., 2014. Regional intestinal drug permeation: biopharmaceutics and drug development. Eur. J. Pharm. Sci. 57, 331–341. https://doi.org/10.1016/j.ejps.2013.08.025.

Lindenberg, M., Kopp, S., Dressman, J.B., 2004. Classification of orally administered drugs on the World Health Organization model list of essential medicines according to the biopharmaceutics classification system. Eur. J. Pharm. Biopharm. 58, 265–278. https://doi.org/10.1016/j.ejpb.2004.03.001.

Lozoya-Agullo, I., Zur, M., Wolk, O., Beig, A., González-Álvarez, I., González-Álvarez, M., Merino-Sanjuán, M., Bermejo, M., Dahan, A., 2015b. In-situ intestinal rat perfusions for human Fabs prediction and BCS permeability class determination: investigation of the single-pass vs. the Doluisio experimental approaches. Int. J. Pharm. 480, 1–7. https://doi.org/10.1016/j.ijpharm.2015.01.014.

Lozoya-Agullo, I., González-Álvarez, I., González-Álvarez, M., Merino-Sanjuán, M., Bermejo, M., 2015a. *In situ* perfusion model in rat colon for drug absorption studies: comparison with small intestine and Caco-2 cell model. J. Pharm. Sci. 104, 3136–3145. https://doi.org/10.1002/jps.24447.

Lozoya-Agullo, I., Zur, M., Beig, A., Fine, N., Cohen, Y., González-Álvarez, M., Merino-Sanjuán, M., González-Álvarez, I., Bermejo, M., Dahan, A., 2016. Segmental-dependent permeability throughout the small intestine following oral drug administration: single-pass vs. Doluisio approach to in-situ rat perfusion. Int. J. Pharm. 515, 201–208. https://doi.org/10.1016/j.ijpharm.2016.09.061.

Lozoya-Agullo, I., Zur, M., Fine-Shamir, N., Markovic, M., Cohen, Y., Porat, D., González-Álvarez, I., González-Álvarez, M., Merino-Sánjuan, M., Bermejo, M., Dahan, A., 2017. Investigating drug absorption from the colon: single-pass vs. Doluisio approaches to in-situ rat large-intestinal perfusion. Int. J. Pharm. 527, 135–141. https://doi.org/10.1016/j.ijpharm.2017.05.018.

Mayer, U., Wagenaar, E., Beijnen, J.H., Smit, J.W., Meijer, D.K.F., Asperen, J.V., Borst, P., Schinkel, A.H., 1996. Substantial excretion of digoxin via the intestinal mucosa and prevention of long-term digoxin accumulation in the brain by the mdr1a P-glycoprotein. Br. J. Pharmacol. 119, 1038–1044. https://doi.org/10.1111/j.1476-5381.1996.tb15775.x.

Neuhoff, S., Yeo, K.R., Barter, Z., Jamei, M., Turner, D.B., Hodjegan, A.M., 2013. Application of permeability-limited physiologically-based pharmacokinetic models: Part I-Digoxin pharmacokinetics incorporating P-Glycoprotein-mediated efflux. J. Pharm. Sci. 102, 3145–3160. https://doi.org/10.1002/jps.23594.

Nielsen, C.U., Abdulhussein, A.A., Colak, D., Holm, R., 2016. Polysorbate 20 increases oral absorption of digoxin in wild-type Sprague Dawley rats, but not in mdr1a(-/-) Sprague Dawley rats. Int. J. Pharm. 513, 78–87. https://doi.org/10.1016/j.ijpharm.2016.09.011.

Nishimura, T., Kato, Y., Amano, N., Ono, M., Kubo, Y., Kimura, Y., Fujita, H., Tsuji, A., 2008. Species difference in intestinal absorption mechanism of etoposide and digoxin between cynomolgus monkey and rat. Pharm. Res. 25, 2467–2476. https://doi.org/10.1007/s11095-008-9658-4.

Oda, K., Murakami, T., 2017. Pharmacokinetic interaction of green tea beverage containing cyclodextrins and high concentration catechins with P-glycoprotein substrates in LLC-GA5-COL150 cells in vitro and in the small intestine of rats in vivo. J. Pharm. Pharmacol. 69, 1736–1744. https://doi.org/10.1111/jphp.12817.

Oga, E.F., Sekine, S., Horie, T., 2013. *Ex vivo* and *in vivo* investigations of the effects of extracts of *Vernonia amygdalina*, *Carica papaya* and *Tapinanthus sessilifolius* on digoxin transport and pharmacokinetics: assessing the significance on rat intestinal p-glycoprotein efflux. Drug Metab. Pharmacokinet. 28, 314–320. https://doi.org/10.2133/dmpk.DMPK-12-RG-117.

Pinto, M., Robine, S., Appay, M.D., 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol. Cell 47, 323–330.

Ruiz-Picazo, A., Lozoya-Agullo, I., Ortiz-Azcarate, M., Merino-Sanjuán, M., González-Álvarez, M., González-Álvarez, I., Bermejo, M., 2017. Comparison of segmental-dependent permeability in human and *in situ* perfusion model in rat. Eur. J. Pharm. Sci. 107, 191–196. https://doi.org/10.1016/j.ejps.2017.06.033.

Schurgers, N., De Blaey, C.J., 1984. Effect of pH, buffer concentration and buffer composition on the absorption of theophylline from the small intestine of the rat. Int. J. Pharm. 19, 283–295. https://doi.org/10.1016/0378-5173(84)90058-9.

Souza, J., Freitas, Z.M.F., Storpirtis, S., 2007. Modelos *in vitro* para determinação da absorção de fármacos e previsão da relação dissolução/absorção. Rev. Bras. Cienc. Farm. 43, 515–527. https://doi.org/10.1590/S1516-93322007000400004.

Souza, J., Benet, L.Z., Huang, Y., Storpirtis, S., 2009. Comparison of bidirectional lamivudine and zidovudine transport using MDCK, MDCK–MDR1, and Caco-2 cell monolayers. J. Pharm. Sci. 98, 4413–4419. https://doi.org/10.1002/jps.21744.

Stappaerts, J., Brouwers, J., Annaert, P., Augustijns, P., 2015. *In situ* perfusion in rodents to explore intestinal drug absorption: challenges and opportunities. Int. J. Pharm. 478, 665–681. https://doi.org/10.1016/j.ijpharm.2014.11.035.

Stephens, R.H., O'Neill, C.A., Warhurst, A., Carlson, G.L., Rowland, M., Warhurst, G., 2001. Kinetic profiling of P-glycoprotein-mediated drug efflux in rat and human intestinal epithelia. J. Pharmacol. Exp. Ther. 296, 584–591.

Suzuki, M., Komura, H., Yoshikawa, T., Enya, S., Nagao, A., Takubo, H., Kogayu, M., 2014. Characterization of gastrointestinal absorption of digoxin involving influx and efflux transporter in rats: application of mdr1a knockout (-/-) rats into absorption study of multiple transporter substrate. Xenobiotica 44, 1039–1045. https://doi.org/10.3109/00498254.2014.920551.

Takara, K., Ohnishi, N., Horibe, S., Yokoyama, T., 2003. Expression profiles of drug-metabolizing enzyme CYP3A and drug efflux transporter multidrug resistance 1 subfamily mRNAS in small intestine. Drug Metab. Dispos. 31, 1235–1239. https://doi.org/10.1124/dmd.31.10.1235.

Tamaki, A., Ierano, C., Szakacs, G., Robey, R.W., Bates, S.E., 2011. The controversial role of ABC transporters in clinical oncology. Essays Biochem. 50, 209–232. https://doi.org/10.1042/BSE0500209.

Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M.M., Pastan, I., Willingham, M.C., 1989. Immunohistochemical localization in normal tissues of different epitopes in the Multidrug Transport Protein P170: evidence for localization in brain capillaries and crossreactivity of one antibody with a muscle protein. J. Histochem. Cytochem. 37, 159–164. https://doi.org/10.1177/37.2.2463300.

Tugcu-Demiroz, F., González-Álvarez, I., González-Álvarez, M., Bermejo, M., 2014. Validation of phenol red versus gravimetric method for water reabsorption correction and study of gender differences in Doluisio's absorption technique. Eur. J. Pharm. Sci. 62, 105–110. https://doi.org/10.1016/j.ejps.2014.05.016.

Ungell, A.L., Nylander, S., Bergstrand, S., Sjöberg, A., Lennernäs, H., 1998. Membrane transport of drugs in different regions of the intestinal tract of the rat. J. Pharm. Sci. 87, 360–366. https://doi.org/10.1021/js970218s.

Ussing, H.H., Zerahn, K., 1951. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. Acta Physiol. Scand. 23, 110–127. https://doi.org/10.1111/j.1748-1716.1951.tb00800.x.

Valenzuela, B., Nácher, A., Casabó, V.G., Martín-Villodre, A., 2001. The infuence of active secretion processes on intestinal absorption of salbutamol in the rat. Eur. J. Pharm. Biopharm. 52, 31–37. https://doi.org/10.1016/S0939-6411(01)00155-2.

Valizadeh, H., Mehtari, M., Zakeri-Milani, P., 2012. Evidence for enhanced intestinal absorption of digoxin by p-glycoprotein inhibitors. Trop. J. Pharm. Res. 11, 939–945. https://doi.org/10.4314/tjpr.v11i6.10.

Van Rees, H., De Wolff, F.A., Noach, E.L., 1974. The influence of diphenylhydantoin on intestinal glucose absorption in the rat. Eur. J. Pharmacol. 28, 310–315. https://doi.org/10.1016/0014-2999(74)90284-2.

Varma, M.V., Gardner, I., Steyn, S.J., Nkansah, P., Rotter, C.J., Whitney-Pickett, C., Zhang, H., Di, L., Cram, M., Fenner, K.S., El-Kattan, A.F., 2012. pH-dependent solubility and permeability criteria for provisional Biopharmaceutics Classification (BCS and BDDCS) in early drug Discovery. Mol. Pharm. 9, 1199–1212. https://doi.org/10.1021/mp2004912.

Varma, M.V.S., Panchagnula, R., 2005. Prediction of *in vivo* intestinal absorption enhancement on P-Glycoprotein inhibition, from rat *in situ* permeability. J. Pharm. Sci. 94, 1694–1704. https://doi.org/10.1002/jps.20309.

Varma, M.V.S., Kapoor, N., Sarkar, M., Panchagnula, R., 2004. Simultaneous determination of digoxin and permeability markers in rat *in situ* intestinal perfusion samples by RP-HPLC. J. Chromatogr. B 813, 347–352. https://doi.org/10.1016/j.jchromb.2004.09.047.

Volpe, D.A., 2010. Application of method suitability for drug permeability classification. AAPS J. 12, 670–678. https://doi.org/10.1208/s12248-010-9227-8.

WHO (2015). WHO Expert Committee on Specifications for Pharmaceutical Preparations. http://apps.who.int/iris/bitstream/10665/81144/1/WHO_TRS_981_eng.pdf (Accessed 01.12.17).

Wilson, T.H., Wiseman, G., 1954. The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. J. Physiol. 123, 116–125. https://doi.org/10.1113/jphysiol.1954.sp005036.

Wu, C.Y., Benet, L.Z., 2005. Predicting drug disposition via application of bcs: transport/absorption/elimination interplay and development of a biopharmaceutics drug disposition classification system. Pharm. Res. 22, 11–23. https://doi.org/10.1007/s11095-004-9004-4.

Yao, H.M., Chiou, W.L., 2006. The complexity of intestinal absorption and exsorption of digoxin in rats. Int. J. Pharm. 322, 79–86. https://doi.org/10.1016/j.ijpharm.2006.05.030.

Zakeri-Milani, P., Valizadeh, H., Tajerzadeh, H., Islambulchilar, Z., 2009. The utility of rat jejunal permeability for biopharmaceutics classification system. Drug Dev. Ind. Pharm. 35, 1496–1502. https://doi.org/10.3109/03639040903037199.

Zaki, N.M., Artursson, P., Bergström, C.A.S., 2010. A modified physiological bcs for prediction of intestinal absorption in drug discovery. Mol. Pharm. 7, 1478–1487. https://doi.org/10.1021/mp100124f.