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Comparison of segmental-dependent permeability in human and *in situ* perfusion model in rat

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ABSTRACT

Nowadays, alternative methods have been developed to predict intestinal permeability values in human as *in vitro*, *in situ* or *ex vivo* methods. They were developed by the necessity to avoid the problems of the human permeability experiments. However, determination of human permeability is needed to properly validate the alternative methods. For this reason, recently, Dahlgren et al. published an indirect method based on a deconvolution technique to estimate the human permeability in different gastrointestinal segments (jejunum, ileum and colon). Therefore, the objective of this research was to demonstrate that Doluisio technique is a useful method to predict the human permeability in different gastrointestinal segments. To achieve this goal, the rat permeability in different segments, of the same drugs studied by Dahlgren et al. (atenolol, metoprolol and ketoprofen), have been compared with the human data obtained by the deconvolution method. The results obtained in this work show that the Doluisio method is a reliable tool to predict segmental human permeability. Consequently, the deconvolution method can be employed to build an extensive database of human permeability, overall from ileum and colon, because there is a lack of human permeability data of these distal segments. Once there are enough human data available, the Doluisio technique will be a valuable alternative method to predict the permeability of new molecules with therapeutic activity without the requirement of human experiments.

1. Introduction

The Biopharmaceutic Classification System (BCS) developed in 1995 by Amidon et al. (1995) establishes the intestinal permeability as one of the most important parameters determining rate and extent of drug absorption (Zur et al., 2014). The parameter employed to quantify drug absorbability is the effective intestinal membrane permeability (P_{eff}). This is a kinetic parameter which reflects the velocity at which a molecule crosses the intestinal barrier and thus it affects the performance of oral drug products in combination with drug solubility and formulation dissolution (Fagerholm, 2008; Sun et al., 2013).

Human P_{eff} is usually determined by *in situ* human intestinal perfusion technique by means of a multi-lumen tube (Loc-I-Gut), which is invasive, has several difficulties and, moreover, it involves the ethical issues associated with human experiments (Dahlgren et al., 2015; Knutson et al., 2009; Lennernas, 2007). For this reason, alternative methods have been developed, as *in vivo* and *in situ* animal models and *in vitro* models with intestinal tissue or cell cultures. However, human intestinal permeability data are required to achieve a strong validation of the alternative method. Until recently, only jejunal human P_{eff} values were available, mainly determined by Lennernas (2007, 2014). Therefore, there is a lack of P_{eff} data from distal gastrointestinal segments, *i.e.* ileum and colon. Knowing the absorption of therapeutic molecules from distal segments is highly relevant to predict the behaviour of controlled release (CR) formulations. To determine human P_{eff} values in distal segments is even a more difficult challenge than in the human jejunum; because of this, recently less invasive methods have been developed. For example, Dahlgren et al. (2016) published in 2016 an indirect method to estimate the human permeability in different gastrointestinal segments (both, in the small intestine and in the large intestine). A deconvolution method, previously validated (Sjogren et al., 2015), is employed to calculate the permeability values. This method uses the

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plasma concentration-time profiles from the tested drug after administering the dissolution in a specific location in the gastrointestinal tract.

Nevertheless, the ideal goal of the permeability studies would be to avoid the use of humans and replace them by alternative methods. This objective will only be achieved when enough human data are available to validate the alternative method. The deconvolution method, mentioned in the above paragraph, allows to obtain easily human permeability data in different segments and consequently it would be the desirable reference for developing and validating preclinical methods. Several works have shown a good correlation between the rat and human small intestine permeability (Cao et al., 2006; Lennernas, 2014; Zakeri-Milani et al., 2007). The perfusion *in situ* techniques in rat intestine are an effective option to study the permeability (Stappaerts et al., 2015). The Doluisio method, developed by Doluisio et al. (1969), is a *in situ* perfusion technique that has demonstrated to be a reliable method to measure the drug permeability in rat small intestine and different intestinal segments, including the colon (Lozoya-Agullo et al., 2015a, 2015b, 2016b, 2017).

The objective of this work was to explore the ability of Doluisio technique to predict the human permeability in different gastrointestinal segments. To achieve this goal, the data obtained in human by the deconvolution method have been compared with the rat permeability of the same model drugs (atenolol, metoprolol and ketoprofen) calculated with the Doluisio method in jejunum, ileum, colon and complete small intestine. Moreover, several correlations have been established between human permeability data and rat permeability data in order to show the suitability of the Doluisio *in situ* perfusion method to estimate human data.

2. Materials and methods

Atenolol, metoprolol and ketoprofen were purchased from Sigma-Aldrich. Methanol, acetonitrile and water were HPLC grade. All other chemicals were of analytical reagent grade.

2.1. Rat permeability studies

The Doluisio studies 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.

The absorption rate coefficients and the permeability values of the three drugs studied were determined in jejunum, ileum, colon and complete small intestine (n = 6–7) using *in situ* "closed loop" perfusion method based in Doluisio Technique (Doluisio et al., 1969) modified to adapt it to each segment as described in previous works (Lozoya-Agullo et al., 2015a, 2016a, 2016b). Briefly, male Wistar rats (body weight, 250–300 g) were anesthetized using a mixture of pentobarbital (40 mg/kg) and butorphanol (0.5 mg/kg). Isolated segments in the jejunum (≈ 45 cm), ileum (≈ 45 cm), colon (≈ 10 cm) and the complete small intestine (≈ 100 cm) were created. In order to remove all the intestinal contents each segment was copiously flushed with a physiologic isotonic solution (1% Sörensen phosphate buffer (v/v), 37 °C). The pH of the isotonic solution was adjusted at the physiological pH of each segment (6.5 for jejunum, 7.4 for ileum, 7.0 for colon and 7.0 for complete small intestine). When the surgical procedure was finished, the abdomen was covered with a cotton wool pad avoiding peritoneal liquid evaporation and heat losses. The drug solution was introduced inside the compartment and the samples were collected every 5 min up to a period of 30 min. Perfused drug solutions were prepared in isotonic saline buffered with Sörensen phosphate buffer (66.6 mM).

At the end of the experiments the animals were euthanized. In order to separate solid components from the samples, they were centrifuged 5 min at 5000 r.p.m. All samples were analyzed by High Performance Liquid Chromatography (HPLC) as described in Table 1 with a previously validated procedure with adequate precision and accuracy and covering the range of the experimental samples.

Table 1
Summary of HPLC conditions for the compounds tested with Doluisio's method. Analytical methods were validated for each compound in terms of specificity, selectivity, linearity, precision and accuracy. All the compounds were analyzed at room temperature, with a flow rate of 1 mL/min and a 150 mm × 4.6 mm C-18 column (5 μm particle size).

Drug	Detection[a]	Mobile phase (aqueous:organic)	λ (nm)	Retention time (min)
Atenolol	F	Water (pH 3): MeOH: MeCN (90:5:5)[b]	231/307[c]	7.0
Metoprolol	F	Water (pH 3): MeOH: MeCN (60:20:20)[b]	231/307[c]	4.3
Ketoprofen	UV	Water (pH 3): MeOH (50:50)[b]	360	4.9

Note: MeCN: acetonitrile, MeOH: methanol.
[a] UV: drug analyzed with an ultraviolet detector, F: drug analyzed with a fluorescence detector.
[b] Water (pH 3) was 0.1% trifluoroacetic acid in water.
[c] λ for excitation/λ for emission.

At the end of the experiments there is a reduction in the volume of the perfused solutions due to water reabsorption, consequently, a correction became necessary to calculate the absorption rate constants accurately. Water reabsorption was characterized as an apparent zero order process. A method based on direct measurement of the remaining volume of the test solution was employed to calculate the water reabsorption zero order constant (k_o). The volume at the beginning of the experiment (V_0) is composed from the volume of the drug solution (4 mL for jejunum and ileum, 5 mL for colon and 10 mL for complete small intestine) plus the residual volume after flushing the intestinal segment. This residual volume was previously characterized and is on average 0.3 to 0.5 mL. The volume at the end of the experiment (V_{end}) was measured for each animal by carefully extracting and squeezing the intestinal segment. An individual value of k_o was estimated for each animal as:

$$k_o = (V_0 - V_{end})/t_{end} \quad (1)$$

where V_{end} is the measured volume at the end of the experiment (t_{end} = 30 min) in each animal. k_o value was used to estimate the remaining water volume in the different segments at each time point (V_t). Finally, the experimental analyzed drug concentrations (C_e) were corrected at each time point to obtain the actual C_t by the following equation:

$$C_t = C_e(V_t/V_0) \quad (2)$$

where C_t represents the drug gut concentration in the absence of any water reabsorption at time t, and C_e represents the actual experimental value. The C_t values (corrected concentrations) were used to calculate the actual 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 (C_t) *versus* time.

$$C_t = C_0 e^{-k_a t} \quad (3)$$

This k_a value was transformed into permeability value with the following relationship:

$$P_{eff} = k_a R/2 \quad (4)$$

where R is the effective radius of the intestinal segment. R was calculated considering the intestinal segment as a cylinder with the relationship:

$$Volume = \pi R^2 L \quad (5)$$

Estimation was done using a 4 mL perfusion volume for jejunum and ileum, 5 mL perfusion volume for colon and 10 mL perfusion

volume for complete small intestine. The intestinal length (L) was 45 cm for jejunum and ileum, 10 cm for colon and 100 cm for complete small intestine.

2.2. Human permeability data

The human permeability data from jejunum, ileum and colon employed to build the figures shown in the results section were taken from Dahlgren et al. (2016).

2.3. Fraction absorbed (F_{abs}) data

In order to establish correlations between the P_{eff} values, from human and rat, and the oral F_{abs} in humans, the following equation was used:

$$F_{abs} = 1 - e^{-P_{eff}\frac{2}{R}T} \quad (6)$$

where R represents the effective radius of the segment perfused according to Eq. (5), and T is the effective absorption time.

3. Results

The permeability values obtained in rat with the perfusion technique based on Doluisio method are shown in Table 2. Table 2 summarized the permeability data in all the rat intestinal segments: jejunum, ileum and colon.

Furthermore, Table 3 shows the rat intestinal permeability using the whole small intestine and the data from the study of Dahlgren et al. (2016) with the human intestinal permeability in jejunum as well as the physicochemical characteristics of the compounds. The Log *P* values of atenolol, metoprolol and ketoprofen have been taken from literature (Lozoya-Agullo et al., 2016b; Shohin et al., 2012).

Figs. 1–4 shows the correlations established between rat permeability data and human Fabs. In the same figures the human permeability values obtained with the deconvolution method by Dahlgren et al. (2016), and their correlation with human Fabs is shown. Fig. 1 refers to the Peff values obtained in complete rat small intestine, Fig. 2 shows the Peff values from jejunum, and Fig. 3 presents the Peff data from rat ileum. Fabs human data have been obtained from literature (Skolnik et al., 2010; Zakeri-Milani et al., 2007).

We are aware that the number of studied compounds is too low to attempt any significant correlation either between rat permeability or human Fabs or between rat Peff and human Peff. For that reason, in order to put in context both datasets and the previous correlations in Figs. 4 and 5, previously published data with both methods (rat and human) in colon and small intestine and their correlations with human oral fraction absorbed have been used to overlap the current data and to show the comparability with previous results. The rat and human permeability data corresponding to these drugs have been taken from previous works (Lozoya-Agullo et al., 2015a; Sjoberg et al., 2013; Tannergren et al., 2009; Zakeri-Milani et al., 2007).

Table 2
Rat permeability values (Peff, cm/s) obtained with Doluisio method. Data presented as average and (SD).

Drug	Jejunum	Ileum	Colon
Atenolol	$2.27{\cdot}10^{-5}$ ($1.80{\cdot}10^{-6}$)	$1.63{\cdot}10^{-5}$ ($1.20{\cdot}10^{-6}$)	$2.11{\cdot}10^{-5}$ ($4.30{\cdot}10^{-6}$)
Metoprolol	$6.85{\cdot}10^{-5}$ ($5.00{\cdot}10^{-6}$)	$9.05{\cdot}10^{-5}$ ($1.23{\cdot}10^{-5}$)	$8.14{\cdot}10^{-5}$($1.87{\cdot}10^{-5}$)
Ketoprofen	$6.95{\cdot}10^{-5}$ ($1.59{\cdot}10^{-6}$)	$3.65{\cdot}10^{-5}$ ($3.31{\cdot}10^{-6}$)	$2.28{\cdot}10^{-4}$($1.49{\cdot}10^{-5}$)

Table 3
Permeability values (Peff, cm/s) from rat complete small intestine and standard deviation (SD) (Doluisio method) and human jejunum (deconvolution method) as well as physicochemical properties of the compounds. *** Data from reference (Dahlgren D., et al).

Drug	Peff (cm/s)		Physicochemical properties***		
	Rat	Human***	MW	Log P	pKa
Atenolol	$1.07{\cdot}10^{-5}$ ($2.40{\cdot}10^{-6}$)	$4.50{\cdot}10^{-5}$	266.3	0.16	9.60[b]
Metoprolol	$6.24{\cdot}10^{-5}$ ($6.46{\cdot}10^{-6}$)	$1.72{\cdot}10^{-4}$	684.8	1.88	9.70[b]
Ketoprofen	$3.00{\cdot}10^{-5}$ ($1.03{\cdot}10^{-6}$)	$8.85{\cdot}10^{-4}$	254.3	3.12	4.45[a]

Note: MW: molecular weight (g/mol).
[a] Acid drugs.
[b] Basic drugs.

4. Discussion

The common human perfusion techniques to study human permeability data from different gastrointestinal segments are characterized by their experimental complexity. However, Dahlgren et al. (2016), developed a new less invasive technique based on a deconvolution method that allows to obtain human permeability values with less experimental challenges.

Nevertheless, once the database of human intestinal permeability values gets big enough, replacing the human experiments by alternative methods is the next desirable goal. The Doluisio rat perfusion method has shown to be a reliable perfusion technique to study the permeability in rat small and large intestine and also in different intestinal segments (Lozoya-Agullo et al., 2015a, 2015b, 2016b, 2017). Therefore, the objective of this work was to evaluate the suitability of the rat and the Doluisio method as a preclinical model to predict the segmental variation in drug permeability of different compounds. To achieve this objective, the permeability of the three model drugs (atenolol, metoprolol and ketoprofen), assayed in humans with the deconvolution method, has been measured in rats with the Doluisio perfusion technique in the same segments studied by Dahlgren et al. (2016): jejunum, ileum and colon. Moreover, the Peff values in the whole rat small intestine have been tested as well. The results of these perfusion experiments in rats can be seen in Table 2.

Until now, the human permeability data available was from jejunum (Lennernas, 2007, 2014), and the number of human Peff determinations in distal human small intestine and human large intestine was limited. Therefore, only the human jejunal Peff values were employed to validate the predictability of the different preclinical intestinal absorption models (including rat) (Dahlgren et al., 2015). The human permeability perfusion methods have been up to date the gold standard to predict the human oral fraction absorbed (Fabs) obtained from pharmacokinetic or mass-balance studies in humans (Dahan et al., 2009; Fairstein et al., 2013; Lennernas, 2007). Obviously the need to validate other preclinical methods to predict human absorption in the development phase requires the comparison against human data.

Figs. 1 to 3 shows a reasonable correlation between rat Peff in the jejunum and ileum segments and human oral Fabs with the same trend displayed by the segmental Peff data in human jejunum and ileum *versus* human Fabs. The phenomenon that the rat values are lower than human values is interesting, and it explains why the rat curves in all Figs. 1 to 3 are to the left of the human ones. However, the reason for the different shape of the curves needs further research. It would be difficult to ensure there is a real mechanistic difference, or if the different shape is due to the low number of assayed compounds. In general, the fact that all the rat curves are to the left of the human ones has been explained for the less effective surface for permeation in the rat small intestine, as this animal lacks of Kerckring folds. The different shape of the curves in the different segments could be explained by a

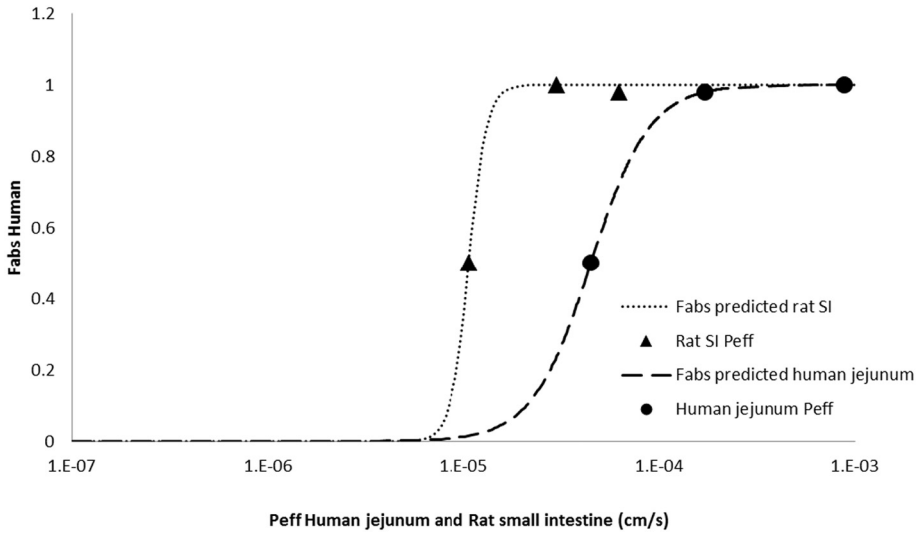

Fig. 1. Correlations between human jejunum permeability and whole rat small intestine (SI) permeability *versus* human fraction absorbed of the three drugs studied in this paper.

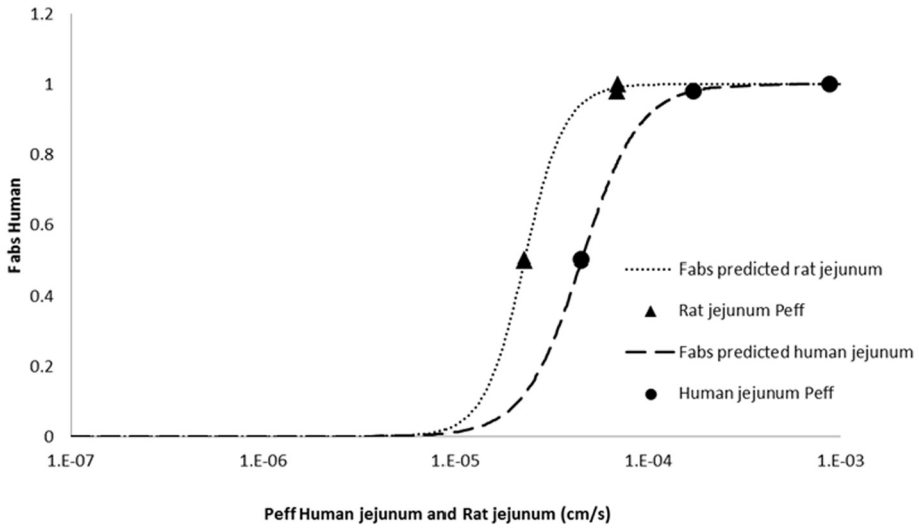

Fig. 2. Correlations between human jejunum permeability and rat jejunum permeability *versus* human fraction absorbed of the three drugs studied in this paper.

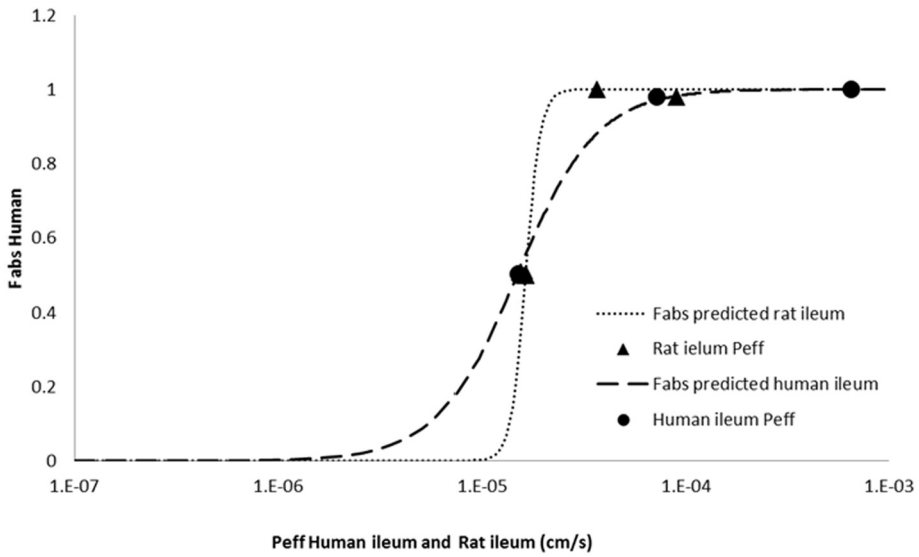

Fig. 3. Correlations between human ileum permeability and rat ileum permeability *versus* human fraction absorbed of the three drugs studied in this paper.

different segmental change of the tight junction's leakiness in rat *versus* human. For the low permeable compounds, in general hydrophilic, a similar to human "tightness" of the junctions would lead to lower permeabilities, making similar the slopes of the curves in Figs. 1 to 3, but as it can be observed in Fig. 3, predicted rat permeabilities on the lower portion of the curves are actually higher than the human ones. That could indicate higher paracellular permeability, as it is observed in colon.

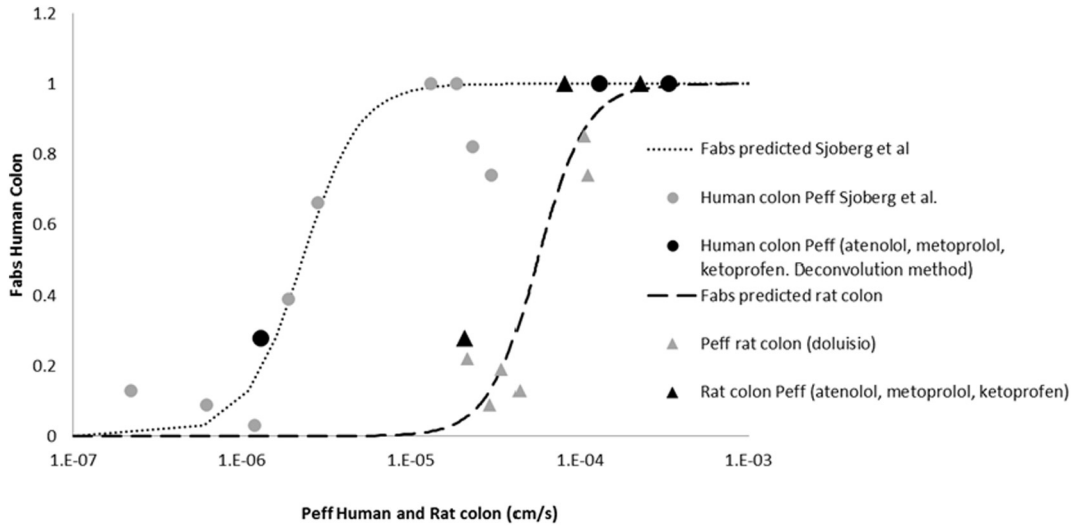

Fig. 4. Correlations between human colon permeability and rat colon permeability *versus* the colonic human fraction absorbed. The black points are the drugs studied in this paper.

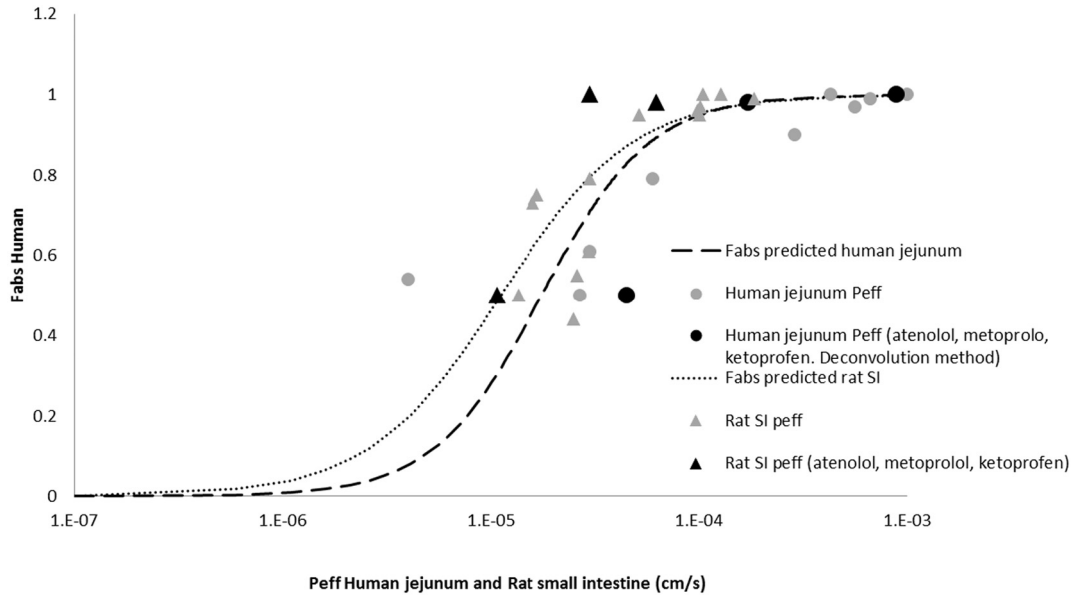

Fig. 5. Correlations between human jejunum permeability and whole rat small intestine (SI) permeability *versus* human fraction absorbed. The black points are the drugs studied in this paper.

Table 4
Goodness of fit indexes of the different Peff *vs* Fabs correlations established with the three drugs studied.

	Fig. 1		Fig. 2		Fig. 3		Fig. 4	
	Human	Rat	Human	Rat	Human	Rat	Human	Rat
RSS	3.1E-08	4.0E-04	3.1E-08	1.8E-04	1.3E-04	4.0E-04	7.8E-02	1.3E-12
AIC	− 47.881	− 19.472	− 47.881	− 21.731	− 22.892	− 19.472	− 1.092	− 50.676
R^2	0.9999	0.9987	0.9999	0.9988	0.9997	0.9987	1.00	1.00

In jejunum and ileum there are few available data in human to compare with the rat experiments so in order to show that the correlations presented are feasible Fig. 4 presents previous correlation of human colon permeability and rat colon permeability *versus* fraction absorbed in human colon, including atenolol, metoprolol and ketoprofen. The figure shows that the current data is in reasonable agreement with our previously published results and maintain the already demonstrated relationship. The general trend can be observed from Fig. 4 is that permeability values from rat colon are higher than those obtained from human colon. This difference has already been seen in a previous work (Lozoya-Agullo et al., 2015a) and it was likely due to the wider tight junctions in rat colon membrane than in human colon membrane.

The goodness of fit indexes of the different correlations established (Figs. 1–4) can be observed in Table 3. All of them have statistical significance ($p < 0.05$), this fact illustrates that both methods, deconvolution (humans) and Doluisio (rats), predict correctly the human absorption in different gastrointestinal segments. Therefore, the Doluisio *in situ* perfusion technique is a useful alternative method to predict human absorption in different gastrointestinal segments, so it can

be employed to avoid human permeability assays.

Table 4 shows the lipophilicity (Log P) and permeability (Peff) human and rat values. Even if a direct correlation is not good and the statistical significance would be limited with just three compounds, those values show that the trend is similar in both data sets and a higher lipophilicity is reflected in higher effective permeability. The good agreement between human data and rat model has been already described in several works (Dahan et al., 2013; Gonzalez-Alvarez et al., 2007; Lozoya-Agullo et al., 2016b; Masaoka et al., 2006; Pham-The et al., 2013). The agreement between permeability values is in particular good in colon. Nevertheless, when comparing across segments in a particular model, the rat presents higher colon permeabilities for a particular compound while in humans the values are similar or lower. On the other hand, Fig. 5 confirms the trend observed in Figs. 1 to 3, which indicate that in general rat intestinal permeabilities are lower that the human ones in jejunum, and thus the rat correlation curve is to the left compared with the human correlation. The reason of that behaviour has been previously explained due to the lower surface available in rat intestine compared to humans as the rat lacks of Kerckring's folders (plicae). In summary Figs. 4 and 5 point out the differences in rat colon *versus* rat small intestine in comparison to the same human segments. In any case with the adequate number of compounds in both models it will be possible to stablish a good correlation and then the conversion factor to predict from the rat model and segment the homologous value in humans.

More human and rat data will be necessary in the future for characterizing the correlation across segmental permeabilities in both models but the data in this work shows that for the jejunum and ileum, the rat shows less differences that the human intestine for that reason the agreement between rat and human is good in jejunum and colon but less good in ileum.

5. Conclusion

The *in situ* perfusion model in rat (Doluisio) has demonstrated to be a reliable tool to predict segmental human permeability in jejunum and colon, thus could be a valuable tool for the development of controlled release drug products.

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