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Mathematical modelling of in situ and in vitro efflux of ciprofloxacin and grepafloxacin

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Abstract

The efflux process due to p-glycoprotein-like mechanisms of ciprofloxacin (CIP) and grepafloxacin (GRX) has been studied "in situ" in rats and "in vitro" in Caco-2 cells. The results were modelled by a curve fitting procedure which allowed the characterization of the passive (P_d) and carrier mediated parameters (V_m and K_m) from the raw data without initial velocities estimation. CIP absorption in rat was characterized as a passive diffusion at the assayed concentrations. Although the involvement of an efflux transporter cannot be ruled out, its relevance in the transport of the fluoroquinolone is negligible. In GRX absorption, an efflux process is implicated and it is detected in both absorption models. GRX permeability depends on the intestinal segment, reflecting the previously reported different expression level of the efflux transporters along the gut in rat. A first attempt to correlate the "in vitro" and the "in situ" data has been done. The mathematical model has been constructed using very simplistic assumptions and it will require further refinement but, nevertheless, the results are promising and demonstrate that a good modelling approach helps to identify the system critical parameters and how the system behaviour change when the parameters are modified as it happens when we move from the "in vitro" to the "in situ" level. Predicted versus experimental permeability values show a good correlation, demonstrating that the relevance of the secretion process "in situ" in rat can be predicted from the "in vitro" cell results.

Keywords: Kinetic modelling; Fluoroquinolones; Intestinal permeability; Caco-2 model; Efflux; Absorption

1. Introduction

Interest in research about the intestinal secretory transport of drugs has grown considerably in recent years. Research has focused on the molecular characterization of the carriers belonging to the ABC family (p-glycoprotein, P-gp; multi-drug resistance system, MRP; and breast cancer resistant protein, BCRP) and on the identification of the drugs interacting with these transporters. The relevance of this secretion process remains unclear since its presence at "in vitro" levels does not correspond always with absorption problems "in vivo" even if some reports have demonstrated an increase in the oral bioavailability of some drugs (i.e. tacrolimus, cyclosporin and paclitaxel) after co-treatment with a P-gp inhibitor (Van Asperen et al., 1998).

The main objective of our study was to test the relevance of the mediated efflux transport in the "in situ" model, in comparison with the "in vitro", and to investigate the physiological distribution of the efflux carrier in the rat intestine from a functional point of view using two fluoroquinolones, ciprofloxacin (CIP) and grepafloxacin (GRX) as model compounds. The relevance "in vivo" of the observed efflux "in vitro" for this class of compounds is still polemic. Both compounds have been studied in "in vitro" models and it has been suggested that both undergo an intestinal secretion process mediated by some Verapamil-sensitive carrier (Hunter et al., 1993; Griffiths et al., 1994; Ramon et al., 1994; Cavet et al., 1997; Yamaguchi et al., 2000). The structural modifications of GRX with respect to the parent compound led to a broader spectrum, longer half-life, larger volume of distribution and increased bioavailability, 72% (Efthymiopoulos, 1997; Efthymiopoulos et al., 1997) versus 50% (Lettieri et al., 1992). Unfortunately GRX was withdrawn from the market due to unexpected and serious adverse reactions. Nevertheless, the compound is a good quinolone

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model for studying the membrane transport mechanism of these antibiotics in order to rationalize the design of new derivatives with improved absorption and bioavailability.

Our main focus is located on investigating modelling procedures as tools for improving the prediction ability of "in vitro" models. With this aim in mind, a non-linear curve fitting method was employed to model the transport processes in both systems and to characterize separately the passive and carrier mediated component. The parameter estimation was done from the concentration versus time profiles or from the cumulative amounts versus time datasets without estimation of initial velocities. This is not the standard procedure in metabolism and transport studies, but it is in agreement with the fact that by definition, Michaelis–Menten equation is a differential equation and nowadays there are available tools for performing the numerical integration of the differential velocity equation.

The results, in terms of estimated parameters, are similar to those obtained by the classical procedures and allow the estimation procedure to be performed in one step and the evolution of concentration (amount) to be predicted in lumen (cell system) at any time point. Finally, we have attempted to predict from the "in vitro" data, the "in situ" permeability values of GRX. The mathematical model has been constructed using a very simplistic assumptions and it will require further refinement but, nevertheless, the results are promising and demonstrate that a good modelling approach helps to identify the system critical parameters and how the system behaviour change when the parameters are modified as it happens when we move from the "in vitro" to the "in situ" level.

2. Materials and methods

2.1. Compounds assayed

CIP was generously donated by Cenavisa (Reus, Spain), and GRX hydrochloride was donated by GlaxoSmithKline (United Kingdom). Verapamil hydrochloride was purchased from Sigma (Barcelona, Spain).

2.2. In situ absorption studies

Male Wistar rats, weighing 200–290 g, were used in the experiments. The "in situ" perfusion ("closed loop") rat gut preparation based on Doluisio et al. method (Doluisio et al., 1969) was adapted as previously described (Martin-Villodre et al., 1986), and performed using the whole small intestine of the rat, or a portion of 33 cm corresponding to proximal, medium or distal fractions from stomach. The bile duct was previously closed with a ligature in order to prevent enterohepatic recycling. This closed loop perfusion technique has been validated in our laboratory as a predictive methodology for drug in vivo absorption (Merino et al., 1995; Sanchez-Castano et al., 2000) and has been widely used to study intestinal absorption yielding realistic transport parameters (Merino et al., 1989; Polache et al., 1991; Martin-Algarra et al., 1994; Ruiz-Balaguer et al., 1997).

Animals were unrestrained in their cages, fasted overnight prior to the experiment and allowed access to water "ad libitum". Surgical procedures were performed under anaesthesia (i.p. administration of 1 g/kg of ethyluretane solution 25% (w/v)). The study was approved by the Scientific Committee of the Faculty of Pharmacy 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 (Real Decreto 223/1988, BOE 67, 18-3-98: 8509–8511).

Test solutions were prepared in isotonic saline adjusted to pH 7.0 with 1% Sörensen phosphate buffer (v/v). Volumes of 10 ml of the test solution were perfused in the assays carried out in the whole intestine of the rat, while 5 ml were used when the experiments were performed in the different fractions.

Four concentrations of CIP were assayed in the whole intestine (1.5, 15, 50 and 150 μM). The highest was selected taking into account the solubility of the compound in the vehicle and the lowest was fixed according to the limit of quantification of the analytical method. In order to assess the possible involvement of a P-gp-like mechanism in the process, the lowest concentration of CIP was also perfused in three segments of the tract, alone and in the presence of verapamil 2 mM.

GRX was studied at two different concentrations (25 and 750 μM) in the whole intestine. To test the influence of P-gp in the secretion process of GRX, two 25 μM solutions, with and without 2 mM of verapamil, were perfused in the three segments. Based on the results from these experiments obtained the proximal segment was selected to perform further experiments. Seven concentrations (2.5, 25, 50, 250, 1250, 2500 and 12500 μM) of GRX were assayed in the proximal segment in order to kinetically characterize the transport.

Samples of the luminal fluid were collected every 5 min for a total of 30 min, and analyzed for their drug content. The apparent absorption rate constants k_{app} were calculated by fitting a first order equation to the drug concentration remaining in lumen versus time data after correcting the concentration for the water reabsorption process as described previously (Doluisio et al., 1970; Martin-Villodre et al., 1986; Valenzuela et al., 2001). This way the remaining concentrations in lumen versus time were used to fit the following first order equation:

$$C = C_0\, e^{-k_{app}t} \quad (0)$$

where the C values are the concentrations remaining in the luminal site at the sampling times t calculated after the water reabsorption correction, k_{app} is the apparent absorption rate constant, and C_0 is the extrapolated drug concentration at time zero.

The intestinal permeability values were calculated taking into account the relationship between k_{app} and P_{eff}: $P_{eff} = (k_{app}R)/2$, where R is the radius of the perfused intestinal segment. The effective intestinal permeability (P_{eff}) of the tested compounds (means of five or six animals) was used as index of the efficiency of absorption.

2.3. In vitro absorption studies

Caco-2 cells were a gift from Dr. M. Hu, Washington State University, USA. Cells were maintained at 37 °C in an

atmosphere of O_2:CO_2 (95:5) and were grown in Dulbecco's modified Eagle's Medium from Sigma (Barcelona, Spain) with $NaHCO_3$ (Panreac Química, Barcelona, Spain) (3.7 g/l), HEPES (Acros Organics, Geel, Belgium) (1.3 g/l) and glucose (3.5 g/l) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 200 mM (1%) L-glutamine, 100 IU/ml Penicillin G, and 100 mg/ml Streptomycin (all from Sigma, Barcelona, Spain). The medium was replaced every 2 days. Cell monolayers were prepared by seeding at a concentration of 25 cells/μl (2 ml total) onto tissue culture inserts (transwell cell culture insert surface 4.2 cm^2, 3 μm pore size (Multiwell™ 6-well, Becton Dickinson, France)). Confluence was reached 7 days after seeding, and the transport experiments were conducted with the monolayers 12–14 days post confluence. The epithelial integrity of the monolayers was checked by measurement of transepithelial electrical resistance (TEER) (Millicel ERS™ device, Millipore, Scharlab, Barcelona). The TEER values obtained ranged from 570 to 750 Ω cm^2. The solution used in all the experiments contained Hank's balanced salt solution (HBSS) (Sigma, Barcelona, Spain) (9.8 g/l) with $NaHCO_3$ (0.37 g/l), HEPES (5.96 g/l) and glucose (3.5 g/l) (Hunter et al., 1991; Hu et al., 1994a; Hu et al., 1994b).

To measure apical-to-basolateral (P_{ab}) and basolateral-to-apical flux (P_{ba}), the test compound was included in the apical and basolateral side, respectively. A shaker device was used to maintain a constant agitation rate (50 rpm) and temperature (37 °C) throughout the experiment. At fixed time points, samples of 0.2 ml were taken from the receptor compartment for analysis of contents and replaced with an equal volume of HBSS.

CIP was assayed at concentrations of 1.5, 50 and 150 μM, while GRX was studied at 25, 50, 250 and 2500 μM.

2.4. Analysis of the samples

An original HPLC procedure was used to quantify the solute concentration in the samples. The method was carried out on a Perkin-Elmer system (Perkin-Elmer, Barcelona, Spain) equipped with a Novapak C18 column (Waters, Barcelona) (3.9 mm × 150 mm). The mobile phase consisted of mixtures of methanol (Scharlau, Barcelona, Spain), acetonitrile (Scharlau, Barcelona, Spain), and an aqueous solution consisting of 15 mM phosphate buffer, adjusted to pH 2.4 (for CIP) or pH 3.0 (for GRX) with orthophosphoric acid. The ratios of these three solvent volumes were 25/5/70 for CIP and 20/20/60 for GRX. Analysis was carried out at 22 °C at a flow rate of 1 ml/min.

Quantification was achieved by fluorometry, with an excitation wavelength of 285 nm and an emission wavelength of 445 nm. The retention time of CIP was 2.40 min and the detection limit was 50 ng/ml. GRX was eluted in 3.25 min and the limit was 25 ng/ml. Both methods were previously validated (Merino et al., 1995; Sanchez-Castano et al., 2000).

Aqueous samples proceeding from the intestinal lumen and from the "in vitro" assays were centrifuged and directly injected into the chromatographic system.

2.5. Statistical analyses

Student's *t*-test and one- or two-way ANOVA analyses followed by a Scheffé or T-3 Dunnet multiple test were performed in order to detect statistical differences between permeability coefficients. All statistical analyses were performed by means of SPSS 10.0 (SPSS Inc., Chicago, USA).

2.6. Fitting of models to data

When the statistical comparison showed variations in the permeability values obtained at different concentrations, the concentration–time data or cumulative amounts versus time data were used to characterize the kinetic model of the secretion process.

The concentration–time data obtained "in situ" were described with a combined kinetics of an active Michaelis–Menten secretion and a passive absorption mechanism. The differential equation describing the model was as follows:

$$\frac{dC}{dt} = \left[-k_d + \frac{V_m}{K_m + C}\right] C \quad (1)$$

where dC/dt represents the change in luminal drug concentration with time (i.e. disappearance due to absorption), k_d corresponds to the passive absorption rate coefficient (from its value was calculated the passive permeability component, P_d, as mentioned above, $P_d = (k_d R)/2$), V_m the maximum transport rate, and K_m the Michaelis–Menten constant (the concentration of substrate at which the transport rate is half of the maximum, $V_m/2$). The V_m parameter obtained was normalized, taking into account the intestinal surface to be compared with the value obtained in Caco-2 cells.

In the "in vitro" assays, the amounts accumulated in the receptor chamber divided by surface area versus time are compatible with a similar equation to that mentioned above, with a passive diffusional component P_d and an active secretion process (V_m and K_m).

The apical-basal (*A* to *B*) and basal to apical (*B* to *A*) data were used to fit Eqs. (2) and (3), respectively.

$$\text{apical to basolateral}: \quad \frac{dQ}{A\,dt} = \left(+P_d - \frac{V_m}{K_m + C}\right) C \quad (2)$$

$$\text{basolateral to apical}: \quad \frac{dQ}{A\,dt} = \left(+P_d + \frac{V_m}{K_m + C}\right) C \quad (3)$$

where *A* stands for the area of the monolayer and the remaining symbols have already been presented.

In order to find the relationship among the "in vitro" and "in situ" permeabilities the following equations were fitted simultaneously to the data

$$\text{apical to basal}: \quad P_{eff} = P_d - \frac{V_m}{K_m + C} \quad (4)$$

$$\text{basal to apical}: \quad P_{eff} = P_d + \frac{V_m}{K_m + C} \quad (5)$$

$$\text{rat}: \quad P_{\text{eff}}^{\text{rat}} = S_{\text{f}}\left(P_{\text{d}} - \frac{V_{\text{m}}}{K_{\text{m}} + C}\right) \tag{6}$$

where P_{eff} is the effective or experimental observed permeability at each concentration and S_{f} is a surface area correction factor.

The underlying assumptions in this model are the following:

(a) The paracellular permeability of GRX is negligible and equivalent in both systems.
(b) The differences in passive permeability (P_{d}) are mainly due to the difference in effective area for transport. An area factor correction (S_{f}) is included to consider this difference (see below).
(c) Affinity for the transporter (K_{m}) and maximal velocity (V_{m}) are assumed to be similar in the whole small intestine. This later point is based on the reported expression levels of P-gp in Caco-2 and in intestinal tissue in rats by other authors (Stephens et al., 2001).

The derivation of the model is described in Appendix A.

All the fitting procedures were carried out by means of Winnonlin v.4.1 software (Pharsight Corp., Palo Alto, USA).

The goodness of fit of the combined model of passive diffusion and saturable efflux was compared with the simple passive diffusion. The indexes of goodness of fit evaluated were the correlation coefficients, r, the AIC (Akaike's information criterion) values and the sum of squared residuals. Snedecor's F-test was used to test the statistical signification of the decrease of the sum of squared residual in the more complex model. Finally, experimental versus predicted permeability values were correlated in order to evaluate the prediction capability of the model.

3. Results and discussion

3.1. Ciprofloxacin

The mean permeability values obtained in the perfusion studies carried out in the whole intestine of the rat at four different concentrations of CIP are listed in Table 1. The one-way ANOVA test did not show significant differences among the conditions assayed. Thus, under these conditions, its absorption can be described as a passive process.

In order to confirm the results and test the influence of the site of absorption, experiments were carried out in various segments, using the lowest concentration of CIP, free of additives and in the presence of the P-gp inhibitor verapamil. Results are also presented in Table 1. Differences were detected using the two-way ANOVA test. Permeability was significantly lower in the proximal segment. Although with no statistical differences, the effect of verapamil was also higher in this part of the intestine.

Multiple efflux systems with distinct substrate specificities depending on the intestinal location have been identified in rat intestine (Saitoh and Aungst, 1995; Emi et al., 1998) and their contribution to fluoroquinolones secretion pointed out (Dautrey et al., 1999; Yamaguchi et al., 2002). Dautrey et al. reported the existence of an efflux process of CIP mediated

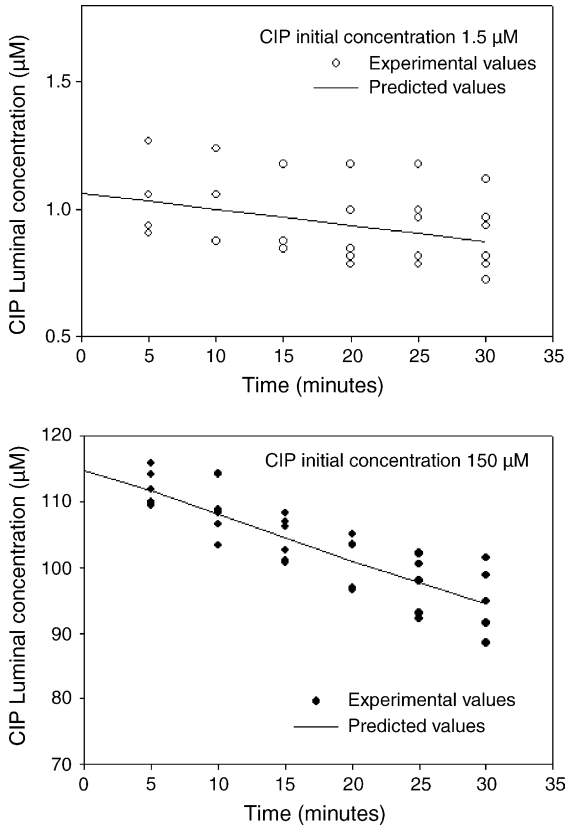

Fig. 1. Examples of concentration of ciprofloxacin in luminal site during absorption assays. Experimental values and fitted lines for the lowest and highest concentrations assayed.

by one of those transporters sensitive to verapamil (Dautrey et al., 1999). Some authors have studied trans-intestinal elimination of CIP after intravenous administration in rats and rabbits (Ramon et al., 1994; Rubinstein et al., 1994) and have found non-linear relationship between the dose of CIP and the amount recovered in the intestinal segment based on a saturable efflux.

Moreover, some authors have demonstrated the existence of a secretion mechanism for CIP and norfloxacin in Caco-2 cell layers (Griffiths et al., 1994; Cavet et al., 1997), which exhibits saturation kinetics and competition with other quinolones.

Therefore, with the results we obtained an efflux process mediated by one transporter sensitive to verapamil cannot be ruled out. Nevertheless, our data show that, at the concentrations used in this study, the active process was not relevant since a first order kinetic can be fitted. As CIP permeability was not dependent on the concentration we assumed that the process could be described as an apparent first order kinetic and Eq. (0) was used to obtain the apparent first order constant. In Fig. 1 the ciprofloxacin experimental and predicted concentrations in lumen versus time at the different initial concentrations are represented. The determinations coefficients of the first order regression was always higher than 0.99.

The fact that the CIP permeability coefficient is similar along the whole intestine can be interpreted as: (a) a consequence of the reduced contribution of the efflux to the global process; or (b) a

Table 1
Ciprofloxacin permeability values and standard deviation obtained in "in situ" perfusion studies and "in vitro" studies (S.D. denotes standard deviation)

Ciprofloxacin permeabilities (cm/s (S.D.))						
Concentration (μM)	Whole intestine	Intestinal segments			Caco-2 cell line	
		Proximal	Medium	Distal	P_{ab}	P_{ba}
1.5	8.25e−6 (3.05e−6)	7.90e−6 (2.18e−6)	1.33e−5 (3.10e−6)	1.65e−5 (7.22e−6)	3.08e−6 (3.71e−7)	6.68e−6 (1.34e−6)
15	1.20e−5 (3.92e−6)					
50	1.20e−5 (2.34e−6)				2.99e−6 (2.92e−7)	5.95e−6 (3.74e−7)
150	1.11e−5 (4.43e−6)				3.32e−6 (3.33e−7)	6.47e−6 (2.36e−7
1.5 + V[a]		1.02e−5 (3.11e−6)	1.41e−5 (3.87e−6)	1.86e−5 (2.89e−6)		

[a] From Rodriguez-Ibanez et al. (2003).

result of other active processes working in the opposite direction (lumen–enterocyte) that would saturate when the concentration is increased, thereby resulting in the same permeability in all the conditions assayed. Neither possibility can be discarded in the light of the results obtained. According to the high variability found in permeability values, the second possibility is feasible (Tartaglione et al., 1986). Nonetheless, we can assume that, given that the addition of verapamil does not produce significant differences, a possible secretion process is irrelevant or insensitive to the said compound. Since the lowest permeability was found in the proximal segment, where the expression of P-gp is lower than down the intestine (Fricker et al., 1996), probably, this transporter is not implied in CIP absorption. Our results agree with those of Lowes and Simmons who demonstrated that neither P-gp nor MRP2 are implicated in CIP secretion (Lowes and Simmons, 2002).

Similar results were obtained using Caco-2 cells. Permeability values of ciprofloxacin in Caco-2 cells are grouped in Table 1. Apical and basal permeability values remained constant when the concentration was altered. In contrast, the ratio basal/apical permeability was higher than 1 (Fig. 2), pointing to a possible secretion of CIP, probably related to one of the ATP-binding cassette proteins. In fact, these ratios reduce to 1 in the presence of verapamil, quinidine (substrate of the OCT 1 transporter) and *p*-aminohippuric acid (substrate of MRP transporters) (Rodriguez-Ibanez et al., 2003). The fact that "in vitro" the effect of verapamil can be evidenced contrarily to "in situ" could be attributed to the highest expression of the transporters in this system.

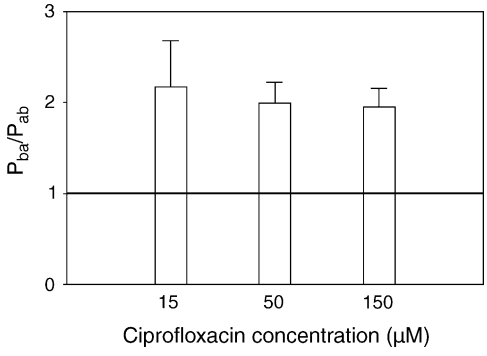

Fig. 2. Ratios between basolateral-apical (P_{ba}) and apical-basolateral (P_{ab}) permeabilities of ciprofloxacin obtained in Caco-2 cells.

3.2. Grepafloxacin

In order to study the linearity in the absorption process of GRX, two concentrations were assayed in the whole intestine of the rat. As illustrated in Table 2, permeability increases significantly as the concentration rises from 25 to 750 μM, suggesting a possible active secretion process. To prevent saturation and to study the regional dependence of this mechanism, the lowest concentration, in the absence and in the presence of 2 mM verapamil, was selected to determine permeability values in three different segments of the small intestine of the rat. Results are summarized in Table 2.

The permeability values of GRX are lower in the proximal and distal segments than in the medium one. The two-way ANOVA test showed significant statistical differences between the permeability values obtained in the different segments. Regional expression of MRP2 transporters is higher in the proximal segment (Stephens et al., 2001), so this carrier could produce a decrease in the net absorption at this site. The lower value for permeability obtained in the distal portion could be explained by the higher expression of P-gp in this portion of the intestine or by physiological factors. It has previously been demonstrated in different animal species that the ratio cholesterol/phospholipids increases along the length of the small intestine, producing a downward increase in the tightness of the epithelium (Kararli, 1995). Thus, there is a decrease in passive diffusion as a consequence of the smaller surface area of the membrane (Ungell et al., 1998).

The addition of verapamil leads to a significant increase of GRX permeability in the three segments. As seen in Fig. 3, the ratio of GRX permeability with/without verapamil is higher in the proximal segment. There is considerable experimental evidence showing that the expression levels of P-gp increase aborally but that the expression levels of MRP2 in rat are higher in the proximal segment (Mottino et al., 2000; Stephens et al., 2001). These facts support the hypothesis that MRP2 could be the main efflux transporter implicated in the secretion of GRX in the rat, although considering our experimental results we cannot rule out the contribution of P-gp, since permeability in the distal portion has the same value. The same results were obtained in male Sprague–Dawley rats by Naruhashi et al. (2002) who concluded that GRX has a secretion process mediated by P-gp and MRP2 and which is greater in the proximal portion of intestine, in accordance with the higher expression levels of MRP2. Par-

Table 2
Grepafloxacin permeability values and standard deviation obtained in "in situ" perfusion studies and "in vitro" studies (S.D. denotes standard deviation)

Concentration (μM)	Grepafloxacin permeabilities (cm/s (S.D.))					
	Whole intestine	Intestinal segments			Caco-2 cell line	
		Proximal	Medium	Distal	P_{ab}	P_{ba}
2.5		3.40e−5 (6.58e−6)				
25	2.03e−5 (4.96e−6)	3.38e−5 (6.22e−6)	4.76e−5 (8.24e−6)	3.11e−5 (3.66e−6)	1.10e−5 (2.41e−7)	2.73e−5 (1.45e−6)
50		3.80e−5 (3.31e−6)			1.28e−5 (3.24e−6)	2.27e−5 (1.24e−6)
250		4.10e−5 (5.12e−6)			1.44e−5 (2.70e−7)	2.45e−5 (1.49e−6)
750	3.82e−5 (4.21e−6)					
1250		5.12e−5 (7.35e−6)				
2500		7.32e−5 (1.05e−5)			2.30e−5 (1.56e−6)	2.13e−5 (1.49e−6)
12500		7.71e−5 (9.66e−6)				
25 + V[a]		5.07e−5 (1.19e−5)	6.16e−5 (1.04e−5)	4.03e−5 (6.71e−6)		

[a] Rodriguez-Ibanez et al. (2003).

allel, Yamaguchi et al. (2002) concluded, using mdr1a/1b(−/−) mice, that grepafloxacin in secreted into the intestine by P-gp.

Since the effect of verapamil was most noted in the proximal segment, different concentrations of GRX were perfused in this part of the intestine so as to evaluate the consequences on absorption. Results are listed in Table 2 where it can be seen that permeability significantly increases as concentration rises. Accordingly, the absorption process of this quinolone is not linear, and there would seem to be a secretory process that acts opposite to passive diffusion, reducing the magnitude of the absorption. The results obtained "in situ" were confirmed "in vitro". In fact, the active process implied in the absorption can also be observed: apical permeability increases and basal values decrease as concentration is raised from 25 to 2500 μM. The ratio basal/apical permeability is higher than one for the three lower concentrations, and the secretory system is probably saturated at the highest concentration—i.e. the ratio basal/apical permeability equals one. We have previously demonstrated in Caco-2 cells that the transporter(s) are sensitive to verapamil, quinidine and ciclosporin A (Rodriguez-Ibanez et al., 2003).

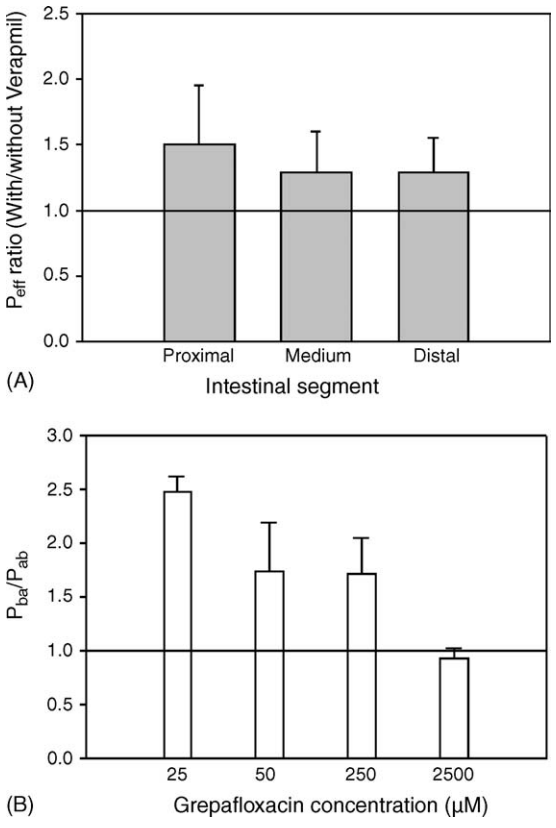

Fig. 3. (A) Ratios between in situ permeability coefficients (P_{eff}) of grepafloxacin in the presence and absence of verapamil obtained in three intestinal segments of rat. (B) Ratios between basolateral-apical (P_{ba}), apical-basolateral (P_{ab}) permeabilities of the compound obtained in Caco-2 cells.

In both systems, "in situ" and "in vitro", we can assume a kinetic that combines a passive diffusion from the lumen to the enterocyte and an active transport in the opposite direction. Kinetic parameters of the combined kinetic model and of the simple passive diffusion model in both experimental systems, "in vitro" and "in situ" as well as the parameters indicatives of the goodness of the fit are shown in Table 3. Either "in vitro" or "in situ" the best fit corresponded to the more complex model that includes diffusion and saturable efflux. The predicted concentrations in the intestinal lumen versus time, as well as the predicted values with both models applied for the higher and lower doses are outlined in Fig. 4 for comparative purposes.

The parameters obtained "in vitro" are very similar to those obtained by Yamaguchi et al. (2000) using the same cells, despite

Table 3
Kinetic parameters of the passive diffusion model and the combined passive diffusion and active efflux model, quantifying grepafloxacin absorption process in situ in rat and in vitro in Caco-2 cells

GRX absorption parameters	In situ studies	In vitro experiments
P_d (cm/s)	4.27e−5 (1.40e−6)	2.5e−5 (7e−7)
WSS	5.76	38.81
R	0.97	0.96
P_d (cm/s)	8.36e−5 (9.49e−9)	2.17e−5 (4.83e−7)
V_m (μmol cm^{-2} s^{-1})	3.1e−5 (1.4e−5)	3.4e−6 (1.7e−6)
K_m (μM)	750 (270)	410 (236)
WSS	3.99	24.22
R	0.994	0.979
F	73.64 (p = 3.4e−29)	6.56 (p = 2.3e−23)

Weighted sum of squared residuals (WSS), correlation coefficients (r) and Snedecor values (F) and their signification (p) are also listed. The numbers in parenthesis indicate standard error of the parameters.

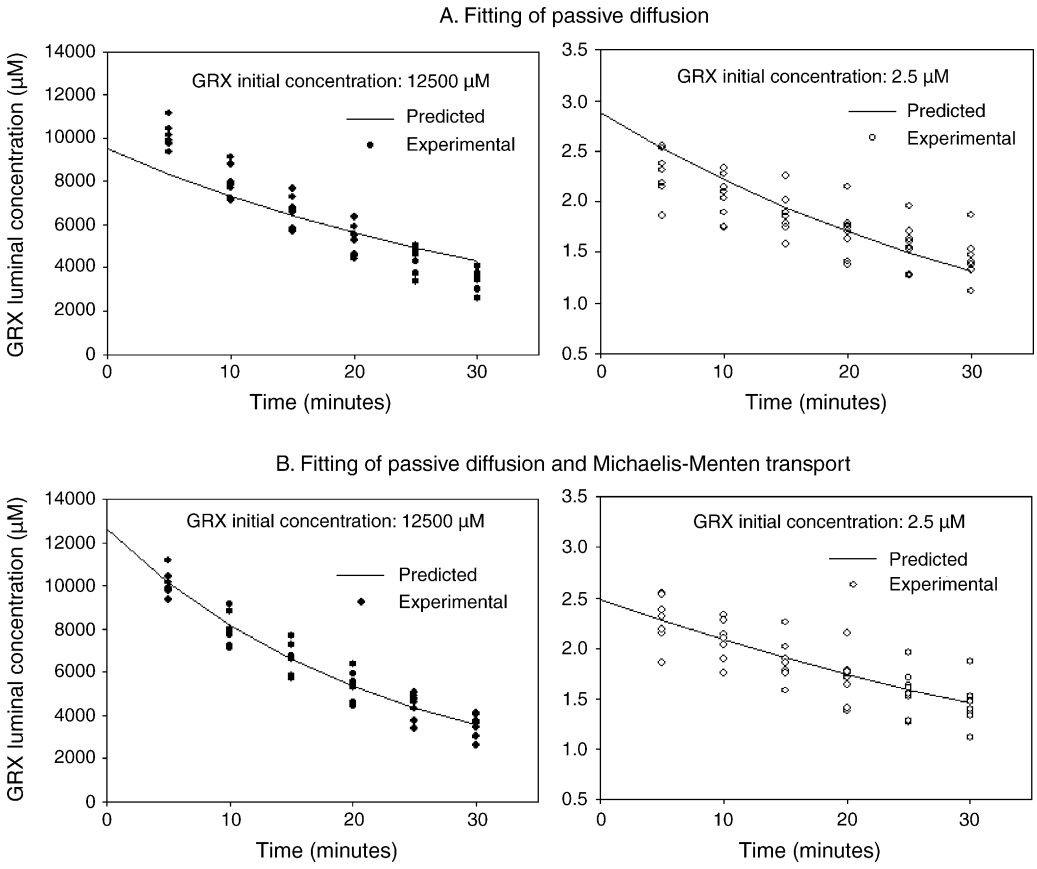

Fig. 4. Examples of concentration of grepafloxacin in luminal site during absorption assays. Experimental values and fitting lines for the lowest and highest concentrations assayed. (A) Assuming passive diffusion and (B) considering passive diffusion and Michaelis–Menten transport.

inter-laboratory variability, but differ from those determined by Lowes and Simmons (2002).

The comparison of the results obtained "in situ" and "in vitro" shows that in both systems it is possible to detect the presence of an efflux process that becomes saturated at the highest concentration. We have assumed that the compound has a minor paracellular permeability in both system based on the molecular weight of the compound and in our previous experience with quinolone absorption in rats (Bermejo et al., 1999). On the other hand, it is possible that either P-gp and MRP2 contribute to the efflux process in both systems and we have considered an equivalent contribution of these transporters. The results of the simultaneous fit of Eqs. (4)–(6) are summarized in Table 4. Data used for fitting the equations were the effective permeability values obtained "in situ" in rat (proximal segment) at different initial GRX concentrations (Table 2 second column) and the apical to basal and basal to apical permeability values obtained in Caco-2 cells (Table 2 fifth and sixth columns). The indexes of goodness of fit are also included in Table 4.

Table 4
Kinetic parameters of the simultaneous fit of "in vitro" and "in situ" permeabilities using Eqs. (4)–(6)

GRX absorption parameters	Parameter value	Standard error	CV%
P_d (cm/s)	1.80e−5	1.00e−6	8.09
V_m (µmol cm^{-2} s^{-1})	1.02e−5	4.90e−6	48.02
K_m (µM)	1000.04	473	47.27
S_f	4.30	0.48	11.18
R^2	>0.95		
SSR	0.166e−9		
AIC	−329.78		

Parameter S_f represents the area correction factor; SSR: weighted sum of squared residuals; AIC: Akaike's information criteria.

The mathematical model used to correlate the "in vitro" and "in situ" permeabilities showed that the area correction factor S_f is around 4 accordingly with the results obtained by other authors (Stewart et al., 1995). This difference is explained by the differences in absorptive surface in the "in situ" versus the "in vitro" model as this later one presents microvilli but not villi or folds. Even if this model has been constructed using very simplistic assumptions, the results are promising and demonstrated that a good modelling approach helps to identify the system critical parameters and how the system behaviour changes from the "in vitro" to the "in situ" level. Predicted versus experimental permeabilities "in vitro" and "in situ" are represented in Fig. 5. It is important to notice that in this plot of predicted versus experimental values we have included in a single linear correlation the values obtained in two different experimental models, "in situ" in rats and "in vitro" in Caco-2 cells, with the single assumption that the main difference between both systems is the actual effective area for transport. The plot is far from being perfect as probably there are more differences in both experimental systems as it could be a slightly different paracellular resistance or different expression levels of the transporter. But even if those

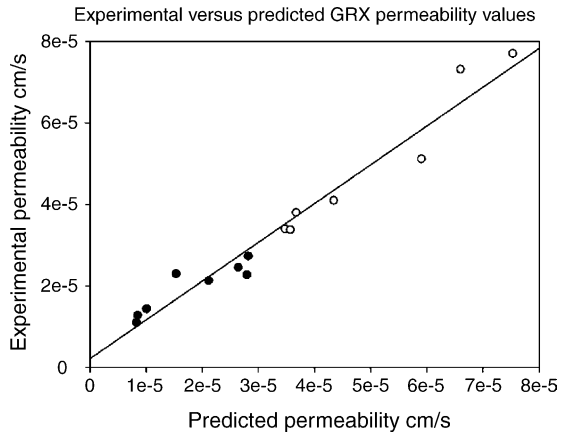

Fig. 5. Experimental vs. predicted permeabilities of grepafloxacin obtained "in vitro" and "in situ" by the simultaneous fit of Eqs. (4)–(6). Black circles correspond to apical to basal and basal to apical GRX permeabilities. White circles are "in situ" permeability data.

factors have not been considered in this preliminary model the results indicate that a simultaneous modelling of "in situ" and "in vitro" data is achievable.

The relevance of a secretion process depends on several factors, such as the affinity for the carrier, its level of expression and the physicochemical characteristics of the drug that determines its passive permeability (Doppenschmitt et al., 1999; Lentz et al., 2000). Our results support the hypothesis that the cell system is not only a good model to reproduce the absorption by passive diffusion in physiological membranes but it could be also used to assess the relevance of the efflux process in the entire animal even if more data will be necessary to obtain a good scaling factor for active processes.

Based in a previously established correlation between bioavailability and permeability in rats for a family of fluoroquinolones (Sanchez-Castano et al., 2000) it is expected that the efflux process could have a minor effect on GRX oral absorption. The lower permeability value obtained "in vitro" and "in situ" (when the functionality of the transporter is maximal) is enough for assuring a fraction absorbed closer to 80%. Actually, the absolute bioavailability determined experimentally in rat in our laboratory at a dose providing much higher luminal concentrations reached this value (so fraction absorbed was at least 80%), so under saturating conditions for the efflux process, other factors limit the complete absorption of the compound.

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Appendix A

A.1. Rationale of model construction

In cell monolayers the experimental observed permeability at each concentration, P_{eff} is calculated from the effective flux, i.e. amount of compound transported by unit time and unit area. The surface area used for the calculations correspond to the area of the insert. As the cells form a monolayer in the insert, this area value does not account for the increase in surface area due to the presence of microvilli.

$$\frac{\mathrm{d}Q}{A\,\mathrm{d}t} = P_{\text{eff}}C$$

where A is the surface area of the monolayer.

In our experimental set up for rat in situ experiments we measure the concentration versus time evolution reflecting the disappearance of the drug from the lumen because of the absorption process. From the absorption rate constants values obtained, permeabilities are calculated using the area/volume ratio. For this calculation we actually use the surface area of the geometrical cylinder A instead of A', the actual surface area available due to Kerkring's fold, villi and microvilli

$$\frac{\mathrm{d}C}{\mathrm{d}t} = k_{\text{a}}C = \frac{A'}{V}P_{\text{eff}}C$$

A' is the surface area available for absorption that includes the increase due to Kerkring's folds, villi and microvilli. This value correspond to A times S_{f}, where A is the surface of the geometrical cylinder and S_{f} is the increase surface factor due to folds and villi.

$$\frac{\mathrm{d}C}{\mathrm{d}t} = \frac{AS_{\text{f}}}{V}P_{\text{eff}}C, \qquad \frac{\mathrm{d}C}{\mathrm{d}t} = \frac{2\pi RL}{\pi R^2 L}S_{\text{f}}P_{\text{eff}}C$$

As we are using an estimation of the intestinal surface area that is lower than the real value, the experimental permeability value in rat is overestimated (i.e. already includes S_{f}).

$$\frac{\mathrm{dC}}{\mathrm{d}t} = \frac{2}{R}S_{\text{f}}P_{\text{eff}}C = \frac{2}{R}P_{\text{eff}}^{\text{rat}}C, \quad P_{\text{eff}}^{\text{rat}} = S_{\text{f}}P_{\text{eff}}$$

This is one of the reasons for obtaining permeability values in rat (human) that use to be 5–10-fold higher than the corresponding values in Caco-2 cells.

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