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Estimation of absorption parameters from the non-steady-state phase in the rat gut perfusion model

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Abstract

The aim of the study was to calculate absorption parameters, including permeability coefficients (P_{app}), from the non-steady-state portion of the outflow to inflow concentration ratio vs time profiles and compare them with those obtained via the more traditionally used steady-state phase. The rat in-situ intestinal perfusion method was used. The compounds studied, diclofenac and macrogol 4000 (polyethylene glycol (PEG) 4000), were perfused at four different flow rates (0.1–2.0 mL min^{-1}). The estimates of P_{app} from the non-steady-state data were systematically lower for both compounds. The non-steady-state analysis gave estimates of the intestinal radius, r. The internal diameter of the intestine segment increased as the flow rate increased. When this effect was taken into account similar P_{app} estimates were obtained by the two approaches. Thus the convention of using a constant value of intestinal radius in the steady-state equation leads to an over estimate of the P_{app} when high flow rates are employed. The different trends observed, between P_{app} and perfusate flow rate, for the two compounds, macrogol 4000 and diclofenac, may be linked to increased surface area and exposure to membrane pores of larger size. The longitudinal spreading coefficient, De, increased with flow rate and was approximately 1000 times greater than that estimated for molecular diffusion. The high values obtained were consistent with the non-smooth biological surface and peristaltic movement present in-vivo.

Introduction

The development of the biopharmaceutical drug classification, which highlights the importance of drug permeability and solubility, and its adoption by regulatory authorities in decisions relating to bio-wavers, has renewed interest in methods for obtaining estimates of drug permeability (Amidon et al 1995). The in-situ single-pass rat intestinal-perfusion technique is widely used to determine drug permeability coefficients (Ho et al 1983; Stewart et al 1997; Barthe et al 1999). It is generally considered superior to the earlier Dolusio method (Dolusio et al 1969), giving better control of hydrodynamics and increased surface area. It is also relatively robust compared with cell culture lines such as CaCo2 cells when exposed to additives such as bile salts (Salphati et al 2001). To estimate the apparent permeability coefficient, P_{app}, (eqn 1), the method relies on the concentration drop between in-flow vs out-flow, at steady state:

$$P_{app} = \frac{-2\pi rl}{Q} * \ln\left(\frac{C_l}{C_0}\right) \quad (1)$$

where C_0 is the input perfusate drug concentration, C_l is the outlet perfusate drug concentration, r is the effective lumenal radius (cm), l is the length of intestine (cm) and Q is the perfusate flow rate (mL s^{-1}). Equation 1 assumes a physical model with complete radial mixing. Alternative mass transport models, which make different assumptions with respect to the experimental convection and diffusion conditions, including laminar flow, plug flow or perfect mixing tank models, may also be applied to determine the permeability coefficient (Amidon et al 1980). In practice different authors have used different perfusion fluid flow rates ranging from 0.1 to 4.0 mL min^{-1} (Ni et al 1980; O'Driscoll & Corrigan 1983; O'Reilly et al 1994; Lane et al 1996).

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This in-situ rat intestinal-perfusion method has been found to correlate with in-vivo human data (Fagerholm et al 1996; Chiou & Barve 1998; Salphati et al 2001). The use of equation 1 requires the attainment of steady state, which in turn is dependent on the fluid flow rate employed. For highly permeable drugs the estimate of P_{app} will be dependent on the fluid flow rate, however there is limited published data on this effect. Ho et al (1983) showed that for progesterone the P_{app} increased with flow rate, while in contrast, no effect was observed in the case of hydrocortisone. With the ionizable compounds, chlorothiazide and salicylic acid, the P_{app} of the former increased with fluid flow while for the latter it appeared to decrease (O'Driscoll 1983). It was suggested that these effects were linked to pH changes affecting the degree of ionization (O'Driscoll & Corrigan 1983). More recently increased fluid flow, resulting in increased pressure, was shown to increase the intestinal diameter and this effect was implicated in the change in absorption observed (Harris & Kennedy 1988; Fihn et al 2000).

Equation 1 allows calculation of the P_{app} in the steady state, which is when the concentration in the effluent becomes constant. The actual concentration vs time profile is in practice sigmoid in shape (Ni et al 1980; Amidon et al 1980; O'Driscoll & Corrigan 1983) and the time to reach steady state decreases with increased fluid flow rate. To estimate the permeability of poorly absorbed drugs, low flow rates may be employed to detect a concentration drop and consequently the time to reach the steady state may be prolonged and difficult to define. In such cases an alternative method of determining P_{app} using the non-steady-state may be feasible. For such purposes a physical model describing the relationship between effluent concentration and time, and incorporating the parameters longitudinal spreading coefficient (De), flow rate (Q), apparent permeability coefficient (P_{app}), intestinal length (l) and radius (r) was developed. It is described using equation 2 (Ni et al 1980):

$$C(x,t)/Co = (e^{\beta x/2\alpha}/2)\{e^{-x\sqrt{(\xi/\alpha)}}\mathrm{erfc}[x/(2\sqrt{(\alpha t)}) - \sqrt{(\xi t)}] + e^{x\sqrt{(\xi/\alpha)}}\mathrm{erfc}[x/(2\sqrt{(\alpha t)}) + \sqrt{(\xi t)}] \quad (2)$$

where:

$$\xi = \beta^2/4\alpha + \gamma,\ \alpha = De,\ \beta = Q/\pi r^2,\ \gamma = 2P_{app}/r$$

C(x,t) is the concentration of drug at any distance x, along the intestine at time t, Co is the concentration of drug at the entrance of the intestinal lumen.

In the steady state equation 2 reduces to equation 3, the form most commonly employed in practice:

$$\frac{C(x,t)}{Co} = \exp(-2\pi r l P_{app}/Q) \quad (3)$$

and, by rearrangement, P_{app} is given by equation 1. Equation 2 introduces the additional parameter, De, which takes account of the flow dependent longitudinal spreading, which occurs before steady state is achieved.

The objective of the current work was to explore the application of equation 2 (non-steady-state) to the determination of P_{app} and the impact of fluid flow on the P_{app} estimates. Non-steady-state profiles were obtained, at a range of fluid flow rates, for three systems; diclofenac, and the marker macrogol 4000 (polyethylene glycol (PEG) 4000), in the presence and absence of diclofenac. Diclofenac (MW 318, pK_a 3.8–4.07, log P 3.09) ionizes in the physiological pH range, it is a non-steroidal anti-inflammatory drug and has been reported to increase gastrointestinal permeability (Yamashita et al 1987). P_{app} values were calculated from the non-steady-state and compared with P_{app} values determined using the more widely used steady-state method. To our knowledge, P_{app} values calculated using the non-steady-state model have not been published previously. In addition, the spreading coefficients (De) for the three systems were determined and compared.

Materials and Methods

Materials

[^{14}C]Macrogol 4000 was obtained from Amersham, Buckinghamshire, UK, and diclofenac from Antigen Pharmaceuticals Ltd, Ireland. All solvents were of HPLC grade. Srensen's phosphate buffer was prepared using Analar grade salts.

Analytical methods

Perfusate concentrations of diclofenac were determined using HPLC as described by El-Sayed et al (1988). Briefly, a 5-μm C-8 reversed phase column and mobile phase of acetonitrile and water (1:1) adjusted to pH 3.3 with glacial acetic acid were used. The flow rate was 2 mL min^{-1} with UV detection at 280 nm. [^{14}C]Macrogol 4000 was analysed by liquid scintillation counting in a Packard TriCarb 2500TR liquid scintillation counter. Quench correction was performed by the method of external standardization.

Animal model

All animal experiments were performed at Trinity College Dublin in association with the BioResources Unit which is registered with the Department of Health, the competent authority designated under EU directive 86/609. The facility is under the full time direction of a veterinary surgeon who maintains the health and welfare programme. Ethical approval was obtained through the veterinary surgeon.

A rat intestinal perfusion technique as described by Komiya et al (1980) was used. Briefly, male Sprague-Dawley rats (250–300 g) were fasted overnight and anaesthetised with pentobarbital sodium before surgery. The intestine was exposed and a segment of the upper small intestine 33.3 cm in length was cannulated at the upper and lower end. Drug solutions were made up in phosphate buffer pH 7.4 and perfused down the intestine at different flow rates (0.1–2.0 mL min^{-1}) controlled by a Braun perfusor pump. All perfusate samples collected were weighed,

and the results indicated no significant change in flow output rate during perfusion.

Statistics and modelling of experimental data

Student's *t*-test was used to test the significance of the difference between two means. Analysis of variance was used when more than two means were compared.

Model parameters were estimated using the non-linear curve fitting and model development program Minsq 4 (Micromath Inc.). Model suitability was assessed using the Model Selection Criterion (MSC) and by residual analysis. MSC allows comparison between various models with different numbers of parameters by relating the coefficient of determination to the number of parameters in the model. This criterion places a burden on the model with the greater number of parameters to have a better coefficient of determination. The most appropriate model is that with the highest MSC.

Results and Discussion

Macrogol data

A number of mathematical models of fluid flow in the intestine have been proposed and used to determine intestinal wall permeability from rat perfusion data. These include the mixing tank model, the laminar flow model, and the plug flow model. The estimated parameters, as determined by the different models, vary according to the assumptions made. Initially the suitability of these models for the system under investigation was assessed in a similar manner to that described by Amidon et al (1980).

The concentration vs time data of the inert marker [^{14}C]macrogol 4000 conducted in rats (n = 4) at 0.5 mL min^{-1} was fitted with the different models (Figure 1). The experimental profile was compared with the theoretical profiles predicted by each model, also shown in Figure 1. The sigmoidal nature of the experimental profile is evident (Figure 1). The profile was similar to that observed by Komiya et al (1980) for the perfusion of progesterone through the rat intestine, and consists of an initial non-steady-state phase on the introduction of the perfusate into the intestine, followed by the steady-state-phase. The non-steady-state model (equation 2) best fitted the profiles obtained for appearance of the tracer molecule in the outflow perfusate, as it described the sigmoidal nature of the profile. This was confirmed by model selection criterion analysis and by residual analysis. Various authors have found that laminar flow adequately models luminal fluid flow in the perfused rat intestine (Elliott et al 1980; Levitt et al 1988). Amidon et al (1980) found the laminar flow model to be the most appropriate of the dimensionless models for the analysis of the perfusion of the macrogol through the rat intestine. They also noted that the tracer molecule appeared in the outflow perfusate somewhat faster than laminar flow would predict, suggesting there was some mixing or dispersion occurring in the system. Such mixing is described by the longitudinal spreading coefficient (De) in the non-steady-state model. The longitudinal spreading coefficient was estimated to be 5×10^{-2} cm^2 s^{-1} for the perfusion of macrogol 4000 through the rat intestine at a flow rate of 0.5 mL min^{-1}. This is of the same order as reported by Ho et al (1983) (2×10^{-2} cm^2 s^{-1}) for the spreading of a non-absorbable marker in the turbulent flow stream along the course of a rubber tube at the same flow rate.

Perfusate flow rate

The effect of flow rate on the absorption parameters, estimated from the non-steady-state model, was then investigated initially using [^{14}C]macrogol 4000. A solution of [^{14}C]macrogol 4000 was perfused through the rat intestine at four different nominal flow rates, ranging from 0.1 to 2 mL min^{-1}. The average percentage of tracer molecule

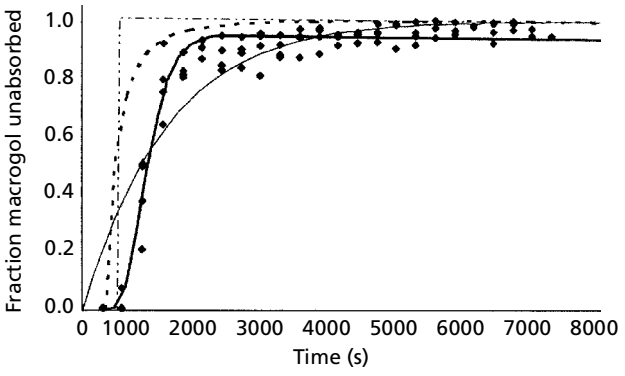

Figure 1 The percentage of macrogol 4000 unabsorbed and that predicted by the non-steady-state model (thick —), the laminar flow model (— —), the mixing tank model (thin —) and the plug flow model (— - —).

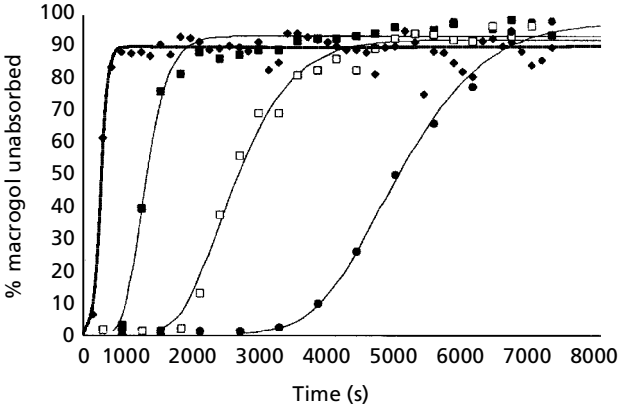

Figure 2 The non-steady-state absorption profile of [^{14}C]macrogol 4000 in a rat gut perfusion model at flow rates of 1.96 mL min^{-1} (◆), 0.49 mL min^{-1} (■), 0.19 mL min^{-1} (□) and 0.08 mL min^{-1} (●) where the points represent the experimental data (n = 4) and the lines the best fit obtained for the non-steady-state model.

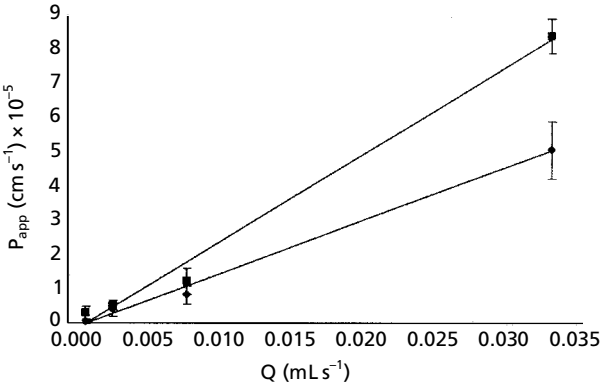

Figure 3 The relationship between the apparent permeability coefficient of macrogol 4000 (± s.d.) (n = 4), estimated from the non-steady-state model (◆) or from the steady-state model (■) and the perfusion flow rate.

in the outflow perfusate to that in the inflow perfusate is given in Figure 2, at each of the four flow rates. It was evident that the time to reach steady state was highly dependent on the fluid flow rate and that the average percent of macrogol in the outflow was less than 100%.

Macrogol 4000 is often included in perfusion solutions as a marker of net gain or loss of water (Komiya et al 1980; O'Reilly et al 1994). It may be employed, as in this study and in that of Amidon et al (1980), to investigate the flow parameters with minimum influence from absorption of the marker. However, absorption of this marker has been reported in the literature when perfused through the rat intestine at flow rates of 0.1 and 0.2 mL min^{-1} (O'Reilly et al 1994; Lane et al 1996). Winne & Gorig (1982) found that up to 4.2% macrogol 4000 was absorbed through the rat jejunum. More recently, Sutton & Rinaldi (2001) reported appreciable absorption ($k_a = 0.002$–0.005 min^{-1}, equivalent to a P_{app} of 0.3–0.9×10^{-5} cm s^{-1}) of [^{14}C]macrogol 3500 from the perfused (0.2 mL min^{-1}) rat intestine.

The decrease in concentration of macrogol 4000 in the outflow perfusate varied with flow rate and was used to estimate the P_{app} at each flow rate. The perfusion rate, as estimated from the perfusion sample weights, and the intestinal length were taken as constants. Estimates of the intestinal radius, longitudinal spreading coefficient and apparent permeability coefficient by a non-linear least squares method are summarized in Table 1. The apparent permeability coefficient, as estimated from the non-steady-state data (eqn 2), was found to increase significantly with increase in flow rate ($P < 0.05$, analysis of variance single factor) (Figure 3). Likewise the apparent permeability coefficient, estimated from the steady-state model (eqn 1), was also found to increase with increase in flow rate (Figure 3). There was a significant difference between the respective estimates of the P_{app} at each flow rate ($P < 0.05$), lower estimates being obtained using the non-steady state method. This difference appears to be related to a change in intestinal radius with flow rate. Lindahl et al (1998) using a rat intestinal perfusion model and a flow rate of 0.2 mL min^{-1} reported a radius of 0.23–0.24 cm in the small intestine. In practice when estimating P_{app} using the steady-state approach the original literature value of 0.18 cm (Komiya et al 1980) is routinely used. In contrast, the non-steady-state analysis gives estimates of the intestinal radius, which are shown to increase significantly with flow rate (Table 1).

The increase in macrogol 4000 intestinal permeability with flow rate (Figure 3) was unexpected as its hydrophilic nature and low membrane permeability would suggest that its permeability should be membrane rather than aqueous boundary layer controlled, and hence independent of flow rate. However Lewis & Fordtran (1975) found that glucose absorption increased 150% when the flow rate increased from 1 to 100 mL min^{-1} and attributed the major effect of enhanced perfusion rate to the increase mucosal surface area, arising from distension of the rat ileum. Harris et al (1988) found that distension led to a reduction in villus height, a marked increase in the width of intervillus space in both transverse and longitudinal dimensions and promoted increased access of luminal contents to intervillus transport sites in the intestine in-vivo. There is evidence also that in the rat the aqueous pore radius varies along the depth of the villus crypt, with small pores at the apical part (< 6Å), larger pores in the crypts (50–60 Å), and medium-sized pores (10–15 Å) in the basal region (Fihn et al 2000). In this study, measurement of the outer diameter of the rat intestine indicated a significant increase from 2.03 to 2.5 cm when the flow rate was increased from 0.1 to 2 mL min^{-1} consistent with distension of the lumen. Thus access to the larger pores is feasible as the flow rate increases.

The longitudinal spreading coefficient increased from 0.0054 to 0.237 cm^2 s^{-1} as the flow rate increased from

Table 1 The absorption parameters estimated for the absorption of macrogol 4000 in phosphate buffer (n = 4) using the non-steady-state absorption model.

	Nominal flow rate (mL min^{-1})			
	2	0.5	0.2	0.1
Q (mL min^{-1}) ± s.d.	1.96 ± 0.04	0.49 ± 0.01	0.19 ± 0.01	0.08 ± 0.01
P_{app} (cm s^{-1} × 10^5) ± s.d.	5.15 ± 0.85	0.824 ± 0.27	0.39 ± 0.19	0.029 ± 0.06
D_e (cm^2 s^{-1}) ± s.d.	0.237 ± 0.06	0.048 ± 0.03	0.021 ± 0.01	0.005 ± 0.002
r (cm) ± s.d.	0.292 ± 0.03	0.279 ± 0.02	0.269 ± 0.01	0.247 ± 0.013

0.08 to 1.96 mL min^{-1} (Table 1). Similar, but lower, increases in the longitudinal spreading coefficients with increase in flow rate through a smooth surfaced rubber tube were reported by Ho et al (1983). The higher values in this study likely reflect the presence of peristaltic movement and the non-smooth nature of the intestine.

Diclofenac and diclofenac plus macrogol 4000

The absorption profiles of diclofenac sodium (1 mg mL^{-1}) in phosphate buffer, pH 7.4, perfused through the rat intestine at four different flow rates are given in Figure 4. Macrogol 4000 was included in these solutions and gave profiles and parameter estimates (for P_{app}, De, r) not significantly different to those obtained in the absence of diclofenac (data not shown). This data suggested that, under the conditions used in this study, diclofenac did not appear to increase intestinal permeability as previously reported (Yamashita et al 1987). The diclofenac absorption profiles were sigmoidal in nature, again showing the initial non-steady-state phase followed by a steady-state phase. The percentage of drug absorbed decreased from 78 to 11% with increase in flow rate from 0.09 to 2.0 mL min^{-1}.

The non-steady-state model parameters, permeability coefficient, longitudinal spreading coefficient, radius and flow rate, were estimated from the diclofenac profiles (Table 2) and the resulting profiles are shown in Figure 4 (solid lines). In addition, P_{app} values for diclofenac were determined using the estimates of De and r obtained from the macrogol 4000 data. These profiles are included in Figure 4 (broken lines). While the latter approach gave a poorer fit, there was no significant difference between the apparent permeability coefficients calculated using parameters from the marker macrogol or from the drug itself, at any of the four flow rates. This is evident from the overlap in the error bars on the plot of P_{app} vs flow rate shown in Figure 5.

As was found with macrogol 4000 (Figure 3), the estimates of P_{app} obtained for diclofenac from the non-steady-state data, were significantly lower ($P < 0.05$) than those estimated using the steady-state model (Figure 5). However, when the apparent change in intestinal radius was taken into account similar estimates were obtained (Table 2). In the case of diclofenac, as the flow rate increased the apparent permeability coefficient tended to increase initially and then appeared to plateau or even decrease (Figure 5).

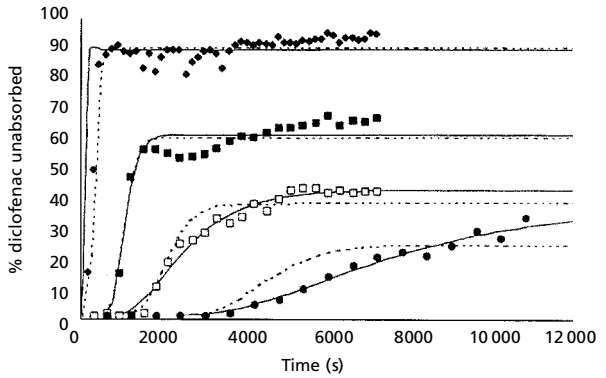

Figure 4 The non-steady-state absorption profile of diclofenac perfused at a flow rate of 2.0 mL min^{-1} (◆), 0.48 mL min^{-1} (■), 0.20 mL min^{-1} (□) and 0.09 mL min^{-1} (●) where the points represent the experimental data, the full lines, the best fit obtained with the non-steady-state model, the broken lines, the best fit obtained with the non-steady-state model, fixing the values of D_e and r to those values previously estimated from the macrogol 4000 perfusion profile.

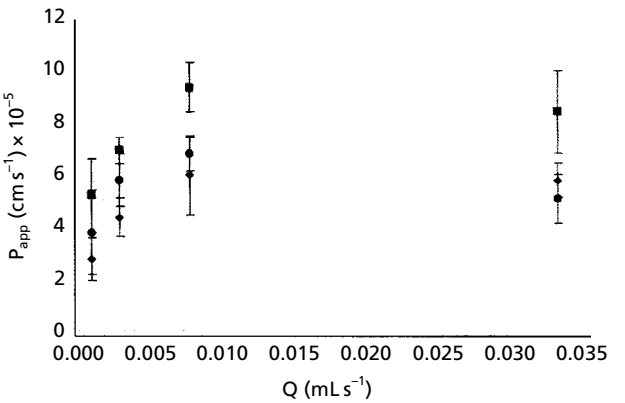

Figure 5 The relationship between the apparent permeability coefficient of diclofenac (± s.d.) (n = 4) estimated from the non-steady-state model (◆), the non-steady-state method using the parameters determined from the perfusion of macrogol (●), the steady-state model (■), and the perfusion flow rate.

Table 2 Comparison of the apparent permeability coefficients for the absorption of diclofenac (n = 4), as estimated from [a]the non-steady-state model using values for D_e and r determined from the diclofenac data, [b]the steady-state model, using a value of 0.2 cm for r, and [c]the steady-state model using r as estimated from the non-steady-state model.

	Nominal flow rate (mL min^{-1})			
	2	0.5	0.2	0.1
Q (mL min^{-1}) ± s.d.	2.00 ± 0.04	0.48 ± 0.01	0.20 ± 0.01	0.09 ± 0.01
[a]P_{app} (cm $s^{-1} \times 10^5$) ± s.d.	5.97 ± 0.65	6.17 ± 1.54	4.56 ± 0.76	2.95 ± 0.84
[b]P_{app} (cm $s^{-1} \times 10^5$) ± s.d.	8.60 ± 1.56	9.55 ± 0.93	7.14 ± 0.52	5.41 ± 1.53
[c]P_{app} (cm $s^{-1} \times 10^5$) ± s.d.	5.91 ± 0.59	6.20 ± 1.37	4.21 ± 1.10	3.25 ± 0.67
D_e (cm^2 s^{-1}) ± s.d.	0.047 ± 0.06	0.077 ± 0.01	0.047 ± 0.06	0.010 ± 0.005

Conclusions

Permeability coefficients were determined from the non-steady-state portion of the percent unabsorbed vs time plots obtained using the in-situ perfusion method. Estimates of P_{app} were systematically lower than the corresponding values obtained using the conventional steady-state method for all three systems investigated: diclofenac, and macrogol 4000 in the absence and presence of diclofenac. The non-steady-state analysis indicated that the internal diameter of the intestine segment increased as the flow rate increased and this increase could explain the different P_{app} estimates obtained by the two approaches. The external diameter of the intestine was found to increase with the perfusate flow rate consistent with the model estimates of internal diameter.

The literature suggested that in addition to increasing surface area, the distension of the intestine would expose lower layers of luminal tissue, having different aqueous pore sizes and hence differing permeabilities, thus altering the estimated apparent permeability coefficient of an individual solute. The different trends observed, between P_{app} and perfusate flow rate, for the two compounds may be linked to these surface area and pore exposure effects.

Plots of log P_{app} vs log flow rate (Q) have been used to distinguish between aqueous boundary layer and membrane-limited transport (Ho et al 1983). The slopes of the log/log plots for both compounds in this study were positive but curved, the slope decreasing as the flow rate increased. This suggested that at low Q there may be a contribution from the aqueous boundary layer for both compounds.

The values of the coefficient for longitudinal spreading (De) calculated were of the order of 1000-times greater than that estimated for molecular diffusion. The estimates of De increased with flow rate and similar values were obtained irrespective of the compounds studied. These values were higher than those reported for a smooth rubber tube and the larger values were consistent with the non-smooth biological surface and peristaltic movement.

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