ELSEVIER

International Journal of Pharmaceutics 155 (1997) 109–119

international journal of pharmaceutics

Evidence of competitive inhibition of methotrexate absorption by leucovorin calcium in rat small intestine

T. Cercós-Fortea, V.G. Casabó, A. Nácher, E. Cejudo-Ferragud, A. Polache, M. Merino *

Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de Valencia, Avda. Vicente Andres Estelles s/n, 46100 Burjassot, Spain

Received 11 April 1997; received in revised form 19 May 1997; accepted 21 May 1997

Abstract

The effect of leucovorin calcium on the intestinal absorption of methotrexate in rat small intestine was investigated using an in situ rat gut technique. First, the kinetic absorption in situ parameters for methotrexate in solution were obtained: $V_m = 21.54$ (± 2.22) μM/h; $K_m = 10.51$ (± 1.08) μM; $k_a = 0.26$ (± 0.03) h^{-1} and AIC = -188.63. The inhibitory effect of leucovorin calcium in methotrexate intestinal absorption has been investigated by perfusing of 10 μM methotrexate isotonic solutions containing increasing concentrations of leucovorin calcium (10–500 μM), and the remaining concentrations of both compounds were measured. A competitive inhibition of methotrexate absorption was detected: the apparent absorption rate constant of the drug decreased as the initial leucovorin calcium concentration increased. Higher leucovorin calcium concentrations, however, did not completely abolish the absorption of the drug (at 500 μM of leucovorin calcium, only 84% inhibition was observed). Apparent parameters characterizing the absorption of leucovorin calcium in the presence of methotrexate 10 μM were: $V_{mi} = 14.70$ (± 1.74) μM; $K_{mi} = 9.43$ (± 1.59) μM; $k_{ai} = 0.28$ (± 0.02) h^{-1}; AIC = -191.53). We can concluded that methotrexate and leucovorin calcium compete for the same intestinal carrier system. This means that since leucovorin calcium, because of its ready conversion to other tetrahydrofolic derivatives (McEvoy, 1996. AHFS Drug Information, Bethesda, MD, pp. 751–758), is administered together with methotrexate in order to prevent the hematopoietic and reticuloendothelial toxic effects of folic acid antagonists, using high leucovorin calcium concentrations, when the urine excretion is decreased, could prevent intestinal drug reabsorption and the drug could then be excreted in the feces, thereby decreasing the risk of poisoning. © 1997 Elsevier Science B.V.

Keywords: Methotrexate; Leucovorin calcium; Intestinal absorption; Michaelis-Menten and first order kinetics; Competitive inhibition

* Corresponding author. Tel.: +34 6 3864912; fax: +34 6 3864911; e-mail: matilde.merino@uv.es

PII S0378-5173(97)00152-X

1. Introduction

Methotrexate is a cytostatic that is extensively used in the treatment of several types of neoplasia, basically osteosarcoma, lymphoblastic acute leukemia, non Hodgkin's lymphoma, pulmonary microcytic cancer and neck and head carcinoma (McEvoy, 1996a). It is a folate antagonist which acts as a potent inhibitor of the enzyme dihydrofolate reductase. A major limiting factor in the use of methotrexate is its toxicity toward nonmalignant, rapidly proliferating tissue, especially that of the small intestine (Bleyer, 1978). Various dosage schedules for methotrexate therapy have appeared in the literature; the dosage, route of administration and duration of therapy must be individualized according to the disease being treated, the other therapies being employed, and the patient's condition, response, and tolerance. Intestinal absorption of methotrexate involves a saturable process and a nonsaturable diffusion process (Said et al., 1985). Methotrexate does not appear to be appreciably metabolized. The drug is excreted primarily by the kidneys via glomerular filtration and active transport. Small amounts are excreted in the feces, probably via the bile. In patients with altered renal function the drug accumulates, thus causing toxicity. Because of enterohepatic circulation this toxicity may be potentiated by prolonged retention of methotrexate in the body (Strum and Leim, 1977; Bleyer, 1978; Shen and Azarnoff, 1978; Steinberg et al., 1982).

Absorption of folates by the intestine represents multiple processes that include transport across the brush-border membrane, travelling across the inside of the enterocytes, and transport across the basolateral membrane. Previous studies in vitro and in vivo have shown that the transport of physiological concentrations of folate ($< 10\ \mu M$) in intact tissue preparations is active, carrier-mediated and pH and Na^+ dependent. Various folate compounds including folic acid, 5-methyltetrahydrofolate and the folate analogue methotrexate share the same transport system but have different affinities (Blair et al., 1974; Strum, 1981; Said and Strum, 1983; Strum and Said, 1983; Said et al., 1984, 1985). Selhub et al. (1984) studied 5-methyltetrahydrofolate transport using rat intestinal loops in vivo and everted jejunal sacs in vitro, and demonstrated the saturability of the transport and the competitive inhibition of 5-methyltetrahydrofolate in methotrexate and folic acid absorption (Selhub et al., 1984). Later Said et al. (1987) found that folic acid transport in human intestinal brush-border membrane vesicles was saturable and that it was inhibited in a competitive manner by the structural analogues 5-methyltetrahydrofolate, methotrexate and 5-formyltetrahydrofolate (leucovorin calcium) (Said et al., 1987). The studies developed by Said and Redha (1987) in basolateral membrane vesicles of rat small intestine also showed that folic acid absorption was saturable as a function of the concentration and inhibited by 5-methyltetrahydrofolate and methotrexate (Said and Redha, 1987). Because leucovorin calcium has an antagonist effect on methotrexate toxicity and the two substances are structural analogues, this folic acid salt was selected and assayed as a possible competitive inhibitor of methotrexate absorption. Methotrexate absorption behaviour in the presence and absence of leucovorin calcium was studied in order to detect possible interactions between the two substances and obtain the kinetic parameters of this interaction. If leucovorin calcium modifies methotrexate intestinal absorption, the intestinal transport of the drug could be inhibited, and then this interaction could be used to prevent methotrexate enterohepatic circulation.

2. Materials and methods

2.1. Absorption studies

All absorption tests were performed on male Wistar rats weighing 250–300 g, fasted for 20 h but with free access to water. Anaesthesia was induced 1 h before surgery by an intraperitoneal injection of ethyl-uretane.

The rat gut in situ preparation (Doluisio et al., 1969), modified as previously reported (Merino et al., 1989; Sánchez-Picó et al., 1989), was used. Briefly, a midline incision was made and the whole small intestine was cannulated. After rins-

ing with physiological saline in order to eliminate fecal residues and debris, 10 ml of a 10 μM isotonized methotrexate solution, in the presence of leucovorin (10, 25, 50, 100 or 500 μM) and buffered to pH 7.0 (Sánchez-Picó et al., 1984), was perfused at 37°C. The sampling intervals were 5 min for a total time of 30 min, and 300 μl of the remaining perfused solution were taken for analysis each time. Six animals per set were employed to study the interaction between methotrexate and leucovorin calcium. These solutions were also used to evaluate luminal disappearance of leucovorin calcium in the presence of the drug.

The in situ technique outlined above was also used to evaluate methotrexate absorption kinetics. In this case, six solutions containing 1, 5, 10, 50, 100 or 1000 μM methotrexate were perfused in the small intestine. Eight animals per set were used for kinetic calculations.

Water reabsorption was evaluated separately for each animal using a method previously reported (Martín-Villodre et al., 1986; Gabus Sannié and Buri, 1987).

2.2. Analytical procedures

Intestinal samples were assayed for methotrexate and leucovorin calcium content by high-performance liquid chromatography (HPLC). The equipment consisted of a Perkin-Elmer Model 10 pump, a variable-volume injector (Rheodyne, P/N 7125-047), and a Perkin-Elmer LC-90 spectophotometric detector. A 150 × 4.6 mm Spherisorb S-5 ODS-2 analytical column (Phase Separations, Ltd., Queensferry) in conjunction with a C-130 B precolumn (Tecknokroma C-18) was used. The mobile phase was a mixture of acetonitrile and aqueous phosphoric acid solution 0.05 M (pH 2.7), 84:16 (v/v), at a flow rate of 1 ml/min. In the 0.05 M phosphoric acid solution the pH was adjusted to 2.7 with HCl 10 N solution.

Intestinal samples were centrifuged at 3000 rpm for 10 min and 50 μl of the solution was injected into the chromatograph. The wavelength used was 303 nm.

Calibration curves covering the entire range of methotrexate and leucovorin calcium concentrations were prepared in triplicate. Excellent linear plots relating the peak area and the concentrations of the two substances were obtained.

The accuracy and precision of the method were validated. The criteria were assessed using six methotrexate concentrations and five leucovorin calcium concentrations covering the entire calibration range of the analytical method (1, 5 10, 50, 100 and 1000 μM in the case of methotrexate and 10, 25, 50, 100, and 500 μM for the leucovorin calcium).

Accuracy was evaluated by calculating the relative error, which was always less than 6%. Precision was evaluated by calculating the coefficient of variation, which was, in all the cases, no higher than 4%. These results were considered completely acceptable (Karnes and March, 1993).

2.3. Fitting of models to data and statistical procedures

2.3.1. Absorption rate measurements

Methotrexate and leucovorin calcium intestinal absorption was quantified using the apparent first-order rate constants, in the usual way, according to the classic expression:

$$A = A_0 \cdot e^{-k_{ap} \cdot t} \quad (1)$$

where A values are the concentrations remaining in the luminal content—already corrected for water reabsorption—at the sampling times, t; k_{ap} is the apparent absorption rate constant and A_o is the initial concentration of the drug, which is always lower than the actual concentration perfused because of membrane adsorption and/or sample dilution (Martín-Villodre et al., 1986). In order to overcome these effects, only the samples obtained between 5 and 30 min were used for calculations (Doluisio et al., 1969; Martín-Villodre et al., 1986; Sánchez-Picó et al., 1989).

Both parameters (A_o and k_{ap}) were then calculated for each animal according to a non-linear regression least-squares procedure.

2.3.2. Absorption kinetics of methotrexate and leucovorin calcium

As a preliminary step, the remaining methotrexate concentrations determined in perfusion fluids for each set of data were fitted to linear kinetics

Table 1
Luminal disappearance of methotrexate[a]

Sampling time (min)	Remaining concentrations of methotrexate (μM) in small intestine for each starting methotrexate concentration (A_i, μM)					
	1	5	10	50	100	1000
5	0.69($\pm$0.06)	3.26($\pm$0.32)	6.34($\pm$0.50)	35.50($\pm$4.67)	66.29($\pm$4.59)	688.1($\pm$31.3)
10	0.57($\pm$0.04)	2.82($\pm$0.32)	5.61($\pm$0.50)	33.72($\pm$4.31)	62.85($\pm$4.59)	672.4($\pm$29.6)
15	0.48($\pm$0.04)	2.41($\pm$0.22)	4.92($\pm$0.43)	31.63($\pm$4.39)	59.38($\pm$4.87)	653.8($\pm$29.1)
20	0.39($\pm$0.04)	2.04($\pm$0.17)	4.20($\pm$0.35)	29.99($\pm$4.32)	56.70($\pm$5.78)	639.1($\pm$31.1)
25	0.33($\pm$0.05)	1.80($\pm$0.21)	3.59($\pm$0.35)	28.18($\pm$4.42)	53.48($\pm$6.37)	624.8($\pm$33.0)
30	0.27($\pm$0.04)	1.46($\pm$0.18)	3.06($\pm$0.34)	26.49($\pm$4.78)	50.95($\pm$6.00)	608.1($\pm$37.0)
k_{ap} (h^{-1})	2.26($\pm$0.29)	1.87($\pm$0.17)	1.72($\pm$0.29)	0.66($\pm$0.22)	0.65($\pm$0.19)	0.30($\pm$0.11)
r	0.999	0.999	0.999	0.999	0.999	0.999

[a] Average concentrations of methotrexate (μM $\pm$ S.D.) remaining in luminal fluid at each methotrexate starting concentration (A_i, μM).

(Eq. (1)) (individual and average values). Absorption rate pseudoconstants were then determined for each initial concentration of methotrexate, as indicated above. The resulting k_{ap} values were statistically compared by means of an ANOVA test in order to detect nonlinearity phenomena in absorption.

Since non-linearities in the methotrexate absorption process were detected, Michaelis-Menten and combined Michaelis-Menten and first order differential equations were fitted to the data:

$$-\frac{dA}{dt}=\frac{V_m\cdot A}{K_m+A} \quad (2)$$

$$-\frac{dA}{dt}=\frac{V_m\cdot A}{K_m+A}+k_a\cdot A \quad (3)$$

Here, dA/dt is the absorption rate of methotrexate (concentration/time), V_m the maximal absorption rate, K_m the Michaelis-Menten constant for methotrexate, k_a the first-order rate constant, and A the methotrexate concentration remaining in the intestinal lumen.

As experimental data, the remaining methotrexate concentrations at each sampling time, shown in Table 1, were used. Calculations were performed globally for all the remaining methotrexate concentrations, that is, for the six sets of data, by using the average values at each time. These fits were performed using the PCNONLIN 3.0 program (Simplex algorithm, weighting factor = $1/A^2$) (Gabrielsson and Weiner, 1994). A differential equation for each starting concentration was used.

The same procedure was used for remaining leucovorin calcium concentrations (Table 3).

2.3.3. Inhibition studies

A clear inhibitory effect of leucovorin on methotrexate absorption was detected and evaluated using a modified form of the Michaelis-Menten equation for complete competitive inhibition with a passive absorption component (Eq. (4)) (Segel, 1975).

$$-\frac{dA}{dt}=\frac{V_m\cdot A}{K_m\cdot\left(1+\frac{I}{K_i}\right)+A}+k_a\cdot A \quad (4)$$

Here, dA/dt is the absorption rate of methotrexate; V_m the maximal absorption rate; K_m the Michaelis-Menten constant for methotrexate; K_i the Michaelis-Menten constant of the inhibitor, and k_a the first-order rate constant. I and A are the inhibitor and drug concentrations remaining in the intestinal lumen. The inhibitor concentration is not a constant since it changes with time according to a Michelis-Menten and first order equation (Eq. (5)).

$$-\frac{dI}{dt}=\frac{V_{mi}\cdot A}{K_{mi}+A}+k_{ai}\cdot A \quad (5)$$

Where, V_{mi} and K_{mi} are the maximal absorption rate and the Michaelis-Menten constant of leucovorin calcium, respectively, and k_{ai} the first order rate constant when this substance acts as inhibitor.

According to this model, the remaining concentrations of methotrexate, A (Table 5), and of leucovorin calcium, I (Table 3), found at each sampling time, t, at the five different initial inhibitor concentrations were used to carry out these fits. They were performed with the PCNONLIN 3.0 program (Simplex algorithm, the inverse of the square of the dependent variable was used as a weighting factor for each datum) using differential equations (Eqs. (4) and (5)) according to the following procedure.

The V_m and K_m values of methotrexate, obtained in free solution, were entered into the program as constants. The rest of the parameters were obtained according to the following procedure:

(1) k_a was entered into the program and its value was the one obtained in the methotrexate kinetic study. Two possibilities were considered:

Model 1: K_{mi} and K_i were considered the same parameter; therefore, the program provided the V_{mi}, K_{mi} and k_{ai} values.

Model 4: K_{mi} and K_i were considered different parameters; therefore, the program provided the V_{mi}, K_{mi}, K_i and k_{ai} values.

(2) The first order rate constant k_a was not entered into the program, and its value was therefore provided by the program. Moreover, we considered two possibilities:

Model 2: K_{mi} and K_i were considered the same parameter; therefore, the program provided the k_a, V_{mi}, K_{mi} and k_{ai} values.

Model 3: K_{mi} and K_i were considered different parameters; therefore, the program provided the k_a, V_{mi}, K_{mi}, K_i and k_{ai} values.

The Akaike information criterion, or AIC (Akaike, 1986), the ANOVA F-test, sum of squares of residuals, SS, and correlation coefficients between theoretical and experimental values, r, were used to assess the goodness of the fits.

3. Results

3.1. Absorption kinetics of methotrexate and leucovorin calcium

The time course of disappearance of the remaining drug concentrations corrected for water reabsorption, is shown in Table 1 for methotrexate and Table 3 for leucovorin calcium. In both cases, absorption rate pseudoconstants, calculated according to Eq. (1), k_{ap}, and correlation coefficients are also given. The ANOVA test performed with the k_{ap} values obtained clearly indicates the existence of non-linearities in methotrexate and leucovorin calcium absorption ($p < 10^{-4}$).

Parameter values and statistical figures (r, SS, AIC) obtained after fitting the Michaelis-Menten differential equation and combined Michaelis-Menten and first-order differential equation (Eqs. (2) and (3)) to the data are shown in Table 2 for methotrexate and in Table 4 for leucovorin calcium.

3.2. Inhibition of methotrexate absorption studies

The average concentrations of methotrexate remaining in the intestinal samples after perfusion of the drug solutions (10 μM) in the presence of variable concentrations of leucovorin calcium,

Table 2
Parameter values ($\pm$ S.D.) obtained after fitting the Michaelis-Menten differential equation (Eq. (2)) and combined Michaelis-Menten and first order differential equation (Eq. (3)) to methotrexate absorption data shown in Table 1

Parameters	Michaelis-Menten	Michaelis-Menten + First order
V_m (μM/h)	(38.64 $\pm$ 3.61)	
K_m (μM)	(17.38 $\pm$ 1.85)	
k_a (h^{-1})		(21.54 $\pm$ 2.22)
A_0 (μM)	(656.94 $\pm$ 5.85)	(10.51 $\pm$ 1.08)
SS	0.0134	(0.26 $\pm$ 0.03)
AIC	-151.34	
$r>$	0.999	

Statistical figures found for each fit are also shown.

Table 3
Luminal disappearance of leucovorin calcium[a]

Sampling time (min)	Remaining concentrations of leucovorin calcium (μM) in jejunal fluid for each starting leucovorin calcium concentration (I_i, μM)				
	10	25	50	100	500
5	6.89($\pm$0.72)	15.27($\pm$1.98)	31.66($\pm$1.47)	67.04($\pm$5.53)	357.4($\pm$33.2)
10	6.17($\pm$0.61)	13.98($\pm$1.53)	30.20($\pm$1.43)	64.14($\pm$4.65)	347.0($\pm$28.3)
15	5.58($\pm$0.59)	12.73($\pm$1.31)	28.58($\pm$1.38)	61.47($\pm$4.28)	333.6($\pm$24.9)
20	5.10($\pm$0.51)	11.90($\pm$1.36)	27.14($\pm$1.55)	58.91($\pm$4.41)	325.1($\pm$22.6)
25	4.51($\pm$0.53)	10.93($\pm$1.14)	25.90 $\pm$ (1.59)	56.65($\pm$4.50)	315.7($\pm$23.6)
30	4.06($\pm$0.52)	9.98($\pm$1.11)	24.60($\pm$1.62)	54.35($\pm$4.65)	305.5($\pm$22.5)
k_{ap} (h^{-1})	1.27($\pm$0.23)	0.98($\pm$0.20)	0.64($\pm$0.09)	0.50($\pm$0.17)	0.37($\pm$0.18)
r	0.999	0.999	0.999	0.999	0.998

[a] Average concentrations of leucovorin calcium (μM, $\pm$S.D.), in presence of constant concentration of methotrexate (10 μM), remaining in luminal fluid at each leucovorin calcium starting concentration (I_i, μM).

corrected for water reabsorption, are shown in Table 5. Absorption rate pseudoconstants, k_{ap}, calculated according to Eq. (1), and correlation coefficients are also given.

Table 6 shows the parameter values (K_i, K_{mi}, k_{ai}, k_a, V_{mi}) and the sum of squares (SS), the r and AIC figures obtained after fitting the complete competitive inhibition plus another component (Eq. (4)) to the data, where I has been replaced by the Michaelis-Menten and first-order combined equation (Eq. (5)) according to the procedure described.

Table 4
Parameter values ($\pm$S.D.) obtained after fitting the Michaelis-Menten differential equation (Eq. (2)) and combined Michaelis-Menten and first order differential equation (Eq. (3)) to leucovorin calcium absorption data in the presence of methotrexate 10 μM shown in Table 3

Parameters	Michaelis-Menten	Michaelis-Menten + First order
V_{mI} (μM/h)	40.56 $\pm$ 5.40	14.70 $\pm$ 1.74
K_{mI} (μM)	27.81 $\pm$ 5.13	9.43 $\pm$ 1.59
k_{aI} (h^{-1})		0.28 $\pm$ 0.02
I_0 (μM)	335.17 $\pm$ 2.86	356.58 $\pm$ 2.41
SS	0.0083	0.0014
AIC	$-$139.85	$-$191.53
$r>$	0.9993	0.9999

Statistical figures found for each fit are also shown.

4. Discussion

4.1. Absorption kinetics of methotrexate

The absorption kinetics of methotrexate has been studied in experimental animals using in vitro and in vivo techniques. Results showed that the intestinal absorption of methotrexate can be described as a saturable process or as a combined mechanism, that is, Michaelis-Menten plus passive diffusion (Strum, 1977, 1981; Strum and Said, 1983; Said and Hollander, 1986). Nevertheless, the study of methotrexate absorption in free solution is necessary in order to gain an insight into its absorption process and to assess the influence of leucovorin calcium on methotrexate absorption and quantify the degree of inhibition.

To detect non-linearities in the methotrexate absorption process it was necessary to use an extensive range of drug concentrations (1000, 100, 50, 10, 5 and 1 μM). Table 1 shows the apparent k_{ap} values obtained along the concentration range used. The classic first-order equation was fitted to the results obtained.

The kinetics of the absorption process can be first-order, Michaelis-Menten or Michaelis-Menten and first order. In the first case, the absorption rate constant, k_a is concentration-independent, while in the second and third cases, it is possible to calculate k_{ap} that is a pseudoconstant

Table 5
Luminal disappearance of methotrexate[a]

Sampling time (min)	Remaining concentrations of methotrexate (μM) in jejunal fluid for each starting leucovorin calcium concentration (A_i, μM)				
	10	25	50	100	500
5	7.06($\pm$0.64)	6.79($\pm$0.62)	6.37($\pm$0.59)	6.87($\pm$0.53)	6.72($\pm$0.50)
10	6.52($\pm$0.68)	6.40($\pm$0.45)	6.14($\pm$0.58)	6.61($\pm$0.51)	6.54($\pm$0.47)
15	5.93($\pm$0.58)	6.00($\pm$0.44)	5.84($\pm$0.48)	6.37($\pm$0.52)	6.40($\pm$0.47)
20	5.38($\pm$0.54)	5.66($\pm$0.34)	5.66($\pm$0.47)	6.14($\pm$0.50)	6.25($\pm$0.46)
25	4.98($\pm$0.54)	5.37($\pm$0.37)	5.47($\pm$0.44)	5.91($\pm$0.52)	6.12($\pm$0.45)
30	4.55($\pm$0.56)	5.10($\pm$0.40)	5.24($\pm$0.46)	5.75($\pm$0.55)	5.98($\pm$0.44)
k_{ap} (h^{-1})	1.07($\pm$0.19)	0.65($\pm$0.01)	0.46($\pm$0.15)	0.43($\pm$0.15)	0.28($\pm$0.08)
r	0.999	0.999	0.999	0.999	0.999

[a] Average concentrations of methotrexate (μM, $\pm$S.D.), remaining in luminal fluid at each leucovorin calcium starting concentration (A_i, μM).

which decreases as the concentration of the drug in the solution increases (Wagner, 1979). Since the apparent first order-constant or pseudoconstant fit methotrexate absorption kinetics well during the 30-min interval of the experiment along the entire range of concentrations used in in situ absorption tests, the changes it undergoes depending on the methotrexate concentration constitute an excellent reference point for determining the existence (if k_a remains constant) or non-existence (if k_a decreases as the methotrexate concentration increases) of linearity. In the latter case the Michaelis-Menten and Michaelis-Menten and first order kinetics should be considered highly probable.

The k_{ap} values tended to decrease as the methotrexate concentration in the perfusion fluid increased. Statistical comparisons, using a one-way ANOVA test, of the k_{ap} values for each set of data showed that these pseudoconstants were statistically different. In order to detect which pairs of k_{ap} values show statistically significant differences, the Scheffé test was used after establishing the probability of false positive errors, i.e. the probability of judging differences to be significant when they are not, at the normal 5% level. The results obtained clearly show that there are no significant differences between the k_{ap} values for the highest drug concentrations (1000 and 100 μM), which means that active transport could be saturated. When concentrations are lower than 10 μM, there are no statistically significant differences between the pseudoconstants. This means that the absorption kinetics of the drug at these concentrations is linear.

Michaelis-Menten and combined Michaelis-Menten and first-order differential equations were fitted to the data on the remaining methotrexate concentrations determined at each sampling time (Eqs. (2) and (3)). The kinetic and statistical parameter values obtained in each case are shown in Table 2.

The fitting of Eqs. (2) and (3) showed similar correlation coefficients between experimental and model-predicted values. Nonetheless, the sum of squares (SS) and Akaike information criterion (AIC) show that the combined Michaelis-Menten and first-order kinetics is the most suitable fitting.

We conclude that a passive component contributes to global absorption of methotrexate, and this contribution is probably significant as compared to the saturable transport mechanism. The limiting value of the latter absorption pathway (i.e. at very low concentration in perfusion fluids, when saturation phenomena are virtually absent, so that k_{ap} should be equal to V_m/K_m) is about 2.22 h^{-1} according to Eq. (3). Consequently, when the carrier – mediated process is far from saturation, the passive component (0.26 h^{-1}) represents 12% of the global absorption rate, whereas

Table 6
Parameter values (± S.D.) obtained after fitting the inhibition model equations to the data

Parameters	Model			
	1 $K_i = K_{mi}$ k_a fixed	2 $K_i = K_{mi}$ k_a free	3 $K_i \neq K_{mi}$ k_a free	4 $K_i \neq K_{mi}$ k_a fixed
V_{mi} (μM/h^{-1})	5.04 ± 0.60	5.04 ± 0.54	13.92 ± 5.22	13.98 ± 6.12
K_{mi} (μM)	0.15 ± 0.01	0.12 ± 0.01	8.59 ± 4.78	8.61 ± 5.61
K_i (μM)			0.12 ± 0.01	0.15 ± 0.01
k_{ai} (h^{-1})	0.42 ± 0.06	0.42 ± 0.05	0.29 ± 0.08	0.29 ± 0.09
k_a (h^{-1})		0.42 ± 0.04	0.43 ± 0.04	
SS	0.0499	0.0372	0.0336	0.0447
AIC	−89.87	−98.68	−100.13	−91.88
$r>$	0.999	0.999	0.999	0.999
$F_{\text{Snedecor (exper)2-3}}$		5.17		
$F_{\text{Snedecor (tab)2-3}}$		4.035		

Statistical figures, AIC and r, obtained for each fit are also shown.

near saturation (0.30 h^{-1}), it represents almost 87% of the total.

4.2. Absorption kinetics of leucovorin calcium

Absorption first-order rate pseudoconstants for leucovorin calcium were determined at concentrations of 10, 25, 50, 100 and 500 μM in the presence of methotrexate 10 μM. As can be observed from Table 3, the absorption rate pseudoconstant significantly decreases as the perfusion concentration increases, thus clearly indicating nonlinearity. As shown in Table 4, statistical figures indicate that the process is governed by combined Michaelis-Menten and first order kinetics. Parameter values are also shown in Table 4, under the combined Michaelis-Menten and first order model.

Obviously, these parameters has been obtained in the presence of methotrexate 10 μM so they could be different from those obtained in free solution (in absence of methotrexate). Nevertheless, due to the differences among concentrations (i.e. the highest leucovorin calcium concentration is fifty fold greater than methotrexate concentrations) we can assume that the V_m, K_m and k_a obtained for leucovorin calcium are practically the actual parameters.

4.3. Inhibition studies

Several studies show that the folate compounds share the same transport systems but have different affinities (Blair et al., 1974; Strum, 1981; Said and Strum, 1983; Strum and Said, 1983; Said et al., 1984, 1985). No available study, however, describes the effect that leucovorin calcium has on methotrexate absorption. Methotrexate reversibly inhibits dihydrofolate reductase, the enzyme that reduces folic acid to tetrahydrofolic acid. Inhibition of tetrahydrofolate formation limits the availability of one-carbon fragments necessary for synthesis of purines and the conversion of deoxyuridylate to thymidylate in the synthesis of DNA and cell reproduction (McEvoy, 1996a). Leucovorin calcium is a derivative of tetrahydrofolic acid. Due to its ready conversion to other tetrahydrofolic acid derivatives, leucovorin is a potent antidote for both the hematopoietic and reticuloendothelial toxic effects of folic acid antagonists (methotrexate, trimethoprim...). As part of a high-dose methotrexate regimen in cancer chemotherapy, leucovorin calcium rescue therapy must begin within 24 h of methotrexate administration (McEvoy, 1996b).

The aim of this paper is characterize the influence of leucovorin calcium on methotrexate intestinal absorption. Therefore, a classic procedure

using the Michaelis-Menten equation properly modified when an inhibition phenomenon exists has been employed. It is important to take into account the fact that since the inhibitor can also be transported, its concentration changes through time as does the drug concentration. The luminal concentrations of the drug and the inhibitor at each sampling time according to the procedure above mentioned, were measured. From the information obtained, we can build a model that reflects the actual absorption process and the inhibition model can be selected better from a statistical point of view. This procedure has been established recently as a good tool to study inhibition phenomenon in the intestinal absorption process (Moll-Navarro et al., 1996). The 10 μM solution was selected for methotrexate inhibition studies because of its similarity with the methotrexate K_m value (Segel, 1975).

Before discussing the results certain considerations should be mentioned. When a compound is absorbed through a single carrier, its K_i value when it acts as an inhibitor should approach its K_m value in free solution (Umeniwa et al., 1979). Whenever the inhibitor is transported by a second carrier which is unable to transport the substrate, K_m becomes a pseudoconstant since it depends on two carrier systems simultaneously, and it can not be equal to K_i. The same reasoning can be applied when the substrate is transported by two carrier systems and the inhibitor is transported by only one of them. Since the inhibitor affects only one part of the drug transport system, K_i and K_m cannot be expected to coincide (Estrada and Hernandez, 1976).

On the other hand, when the intestinal transport of a compound is described as Michaelis-Menten and first-order kinetics and a structural related compound (inhibitor) inhibits completely its mediated transport, the k_a (first order rate) value of the substrate obtained in the kinetics and inhibition models should be the same. If the k_a value obtained from the inhibition model is bigger than the one obtained in the kinetic model, it is because the drug is still being absorbed by active transport, and this mean that the active transport is not completely inhibited.

The reported results clearly show that k_{ap} values, take as a functional absorption index for methotrexate, significantly decrease to a limiting value of (0.28 ± 0.08 h^{-1}) (ANOVA $p < 0.01$) as the leucovorin calcium concentration increase, as shown in Table 5. This behaviour is due to the absorption kinetics of methotrexate since the process is carried out by a carrier-mediated component and a passive one. The latter might be responsible for the residual absorption rate constant. The active process could be completely inhibited and the passive process could be operative in the presence of high concentrations of leucovorin.

The inhibition model represented by Eqs. (4) and (5) were used for characterising the nature of the interaction between the two substances.

It is important to emphasize that in the inhibition model the V_m and K_m parameter values of methotrexate can not be estimated separately with accuracy because only the results from only one concentration of methotrexate (10 μM) are available, and in these cases, a striking interdependence between V_m and K_m arises (Reich, 1981).

The statistical parameters reported in Table 6 are indicative of the goodness of each fit. The sum of squares values and the AIC test actually showed that Model 3 was the best. This was confirmed by the Snedecor F-test, with the aid of which significant differences were found ($F = 5.17$, $p < 0.05$).

According to the data obtained, we can reasonably conclude that methotrexate and leucovorin calcium absorption in rat small intestine is mediated by more than one carrier systems (K_i and K_{mi} values were different) and these carriers are shared by the two substances.

However, the leucovorin calcium kinetic parameters V_{mi} and K_{mi} obtained in the inhibition model (Table 6) have a large standard deviation, which could cast doubt on its suitability, but if we compare these values with the ones obtained in the kinetic model (Table 4) we find that the values coincide. This suggests that the problem with the standard deviation is caused by the large number of parameters that have to be calculated, but not due to the inadequacy of the model.

On the other hand, k_a value obtained in the inhibition model (0.43 h^{-1}) is higher than that obtained in the kinetic model (i.e. in free solution) (0.26 h^{-1}). The Student's t-test confirms that there is statistically significant differences between this constants, so we can concluded that methotrexate mediated transport would not be completely inhibited by high concentrations of leucovorin calcium.

These results seem to agree with those reported by other author (Zimmerman, 1992). They suggest the existence of two pathways of intestinal transport of methotrexate, one of which is shared with folic acid while the other is not shared with folic acid and may be responsible for the major part of the methotrexate transported across the mucosa of the human small intestine.

4.4. Biopharmaceutical implications

From a clinical point of view, interactions which influence the effectiveness and safety of therapy are relevant, particularly in the management of certain pathologies. Possible practical implications arising from methotrexate and leucovorin calcium interaction at the absorption level should be analyzed bearing in mind not only the magnitude of the inhibitory effect but also the fact that the presence of leucovorin calcium at high concentrations decreases the methotrexate bioavailability.

Methotrexate is excreted by kidney via glomerular filtration and active transport, and a small amount is excreted in the feces via the bile. In patients with altered renal function the drug accumulates because enterohepatic circulation of the drug induces an increase in the half life.

From the results obtained in the present study, it can be deduced that the interaction between methotrexate and leucovorin calcium may be clinically relevant because the inhibitor effect could prevent reabsorption of the drug excreted via the bile, thus improving the elimination of the drug in the feces.

According to our results, the usefulness of leucovorin calcium to decrease methotrexate toxicity by inhibiting its intestinal absorption depends on the ratio concentrations of both compounds in the luminal content. Bearing in mind that Michaelis-Menten constant, K_m, obtained for both compounds is similar (10.51 ± 1.08 μM for methotrexate and 9.43 ± 1.59 μM for leucovorin calcium), it would be necessary that leucovorin calcium concentration was, at least, double that methotrexate concentration to obtain a significant inhibition of the intestinal transport of methotrexate.

In conclusion, the interaction described here could lead to a new dosage schedule for leucovorin calcium that would decrease the reabsorption of the methotrexate in the small intestine preventing its toxicity when a renal failure exists.

References

Akaike, A., 1986. An information criterion (AIC). Math. Sci. 14, 5–9.

Blair, J.A., Johnson, L.T., Matty, A.J., 1974. Absorption of folic acid everted segments of rat jejunum. J. Physiol. Lond. 236, 653–661.

Bleyer, W.A., 1978. The clinical pharmacology of methotrexate. Cancer 41, 36–51.

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. 56, 1196–1200.

Estrada, E., Hernandez, M., 1976. Portadores múltiples. In: Blume. H.M. (Ed.), Cinética de Transporte a Través de Membranas. Madrid, pp. 127–145.

Gabrielsson, J., Weiner, D., 1994. PK/PD Data Analysis: Concepts and Applications. Swedish Pharmaceutical Press, Stockholm, pp. 9–13.

Gabus Sannié, C., Buri, P., 1987. Étude comparative des méthodes de détérmination du volume d'eau absorbé lors de perfusion de l'intestine grêle du rat. S.T.P. Pharma. 3 (11), 856–860.

Karnes, H.T., March, C., 1993. Precision, accuracy, and data acceptance criteria in biopharmaceutical analysis. Pharm. Res. 10, 1420–1426.

Martín-Villodre, A., Plá-Delfina, J.M., Moreno-Dalmau, J., Pérez-Buendía, M.D., Miralles, J., Collado, E., Sánchez-Moyano, E., Del Pozo, A., 1986. Studies on the reliability of a bihyperbolic functional absorption model. I. Ring substituted anilines. J. Pharmacokinet. Biopharm. 14, 615–633.

McEvoy, G.K., 1996. Antineoplasic agentes. In: AHFS Drug Information. Bethesda, MD, pp. 751–758.

McEvoy, G.K., 1996. Unclassified therapeutic agentes. In: AHFS Drug Information. Bethesda, MD, pp. 2743–2746.

Merino, M., Peris-Ribera, J.E., Torres-Molina, F., Sánchez-Picó, A., García-Carbonell, M.C., Casabó, V.G., Martín-

Villodre, A., Plá-Delfina, J.M., 1989. Evidence of a specialized transport mechanism for the intestinal absorption of baclofen. Biopharm. Drug Dispos. 10, 279–297.

Moll-Navarro, M.J., Merino, M., Casabó, V.G., Nácher, A., Polache, A., 1996. Interaction of taurine on baclofen intestinal absorption: a nonlinear mathematical treatment using differential equations to described kinetic inhibition models. J. Pharm. Sci. 85 (11), 1248–1254.

Reich, J.G., 1981. On parameter redundancy in curve fitting of kinetic data. In: Endreny, L. (Ed.), Kinetic Data Analysis: Design and Analysis of Enzyme and Pharmacokinetics Experiments. Plenum Press, New York, pp. 39–50.

Said, H.M., Strum, W.B., 1983. A pH-dependent, carrier-mediated system for transport of 5-methyltetrahydrofolate in rat jejunum. J. Pharmacol. Exp. Ther. 226, 95–99.

Said, H.M., Hollander, D., Katz, D., 1984. Absorption of 5-methyltetrahydrofolate in rat jejunum with intact blood and lymphatic vessels. Biochim. Biophys. Acta 775, 402–408.

Said, H.M., Ghishan, F.K., Murrell, J.E., 1985. Ontogenesis of the intestinal transport of 5-methyltetrahidrofolate in the rat. Am. J. Physiol. 249 (Gastrointest. Liver Physiol. 12), G567–G571.

Said, H.M., Hollander, D., 1986. Inhibitory effect of bile salts on the enterohepatic circulation of methotrexate in the unanesthetized rat inhibition of methotrexate intestinal absorption. Cancer Chemother. Pharmacol. 16, 121–124.

Said, M., Ghishan, F.K., Redha, R., 1987. Folate transport by human intestinal brush border membrane vesicles. Am. Physiol. Soc. 252, G229–236.

Said, H.M., Redha, R., 1987. A carrier mediated transport for folate in basolateral membrane vesicles of rat small intestine. Biochem. J. 247, 141–146.

Sánchez-Picó, A., Torres-Molina, F., Martín-Villodre, A., Domenech, J., 1984. Plá-Delfina, J.M., 1984. Contribución al estudio de las ventanas de absorción del baclofen en distintos tramos del intestino de rata. Second Eur. Cong. Biopharm. Pharmacokinet., Salamanca 2 (1984), 252–260.

Sánchez-Picó, A., Peris-Ribera, J.E., Toledano, C., Torres-Molina, F., Casabó, V.G., Martín-Villodre, A., Plá-Delfina, J.M., 1989. Nonlinear intestinal absorption kinetics of cefadroxil in the rat. J. Pharm. Pharmacol. 41, 179–185.

Segel, H.I., 1975. Enzymes Kinetics. Wiley, New York.

Selhub, J., Powell, G.M., Rosenberg, I.H., 1984. Intestinal transport of 5-metiltetrahidrofolate. Department of Medicine, American Physiological Society, pp. G515–520.

Shen, D.D., Azarnoff, D.L., 1978. Clinical pharmacokinetics of amethopterin. Clin Pharmacol. 3, 1–13.

Steinberg, S.E., Campbell, C.L., Bleyer, C.L., et al., 1982. Enterohepatic circulation of methotrexate in rats in vivo. Cancer Res. 42, 1279–1282.

Strum, W.B., 1977. A pH-dependent, carrier-mediated transport system for the folate analogue amethopterine in rat jejunum. J. Pharmacol Exp Ther. 203, 640–645.

Strum, W.B., Leim, H.H., 1977. Hepatic uptake, intracellular protein binding and biliary excretion amethopterin. Biochem Pharmacol. 26, 1235–1240.

Strum, W.B., 1981. Characteristics of the transport of pteroylglutamate and amethopterin in rat jejunum. J. Pharmacol. Exp. Ther. 216, 329–333.

Strum, W.B., Said, H.M., 1983. Intestinal folate transport. A pH-dependent, carrier mediated process. In: Blair, J.A. (Ed.), Chemistry and Biology of Pteridines. de Gruyter, Berlin, pp. 1019–1023.

Umeniwa, K., Ogino, O., Miyazaki, K., Arita, T., 1979. Intestinal absorption of several beta-lactam antibiotics. II. Absorption characteristics of amino-penicillins and amino-cephalosporins. Chem. Pharm. Bull. 27, 2177–2182.

Wagner, J.G., 1979. Fundamentals of Clinical Pharmacokinetics. Drug Intelligence Publ., Hamilton, ON.

Zimmerman, J., 1992. Methotrexate transport in the human intestine: evidence for heterogeneity. Biochem. Pharmacol. 43, 2377–2383.

.