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Adsorption of methotrexate and calcium leucovorin onto cholestyramine in vitro

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Abstract

Methotrexate (MTX), an antimetabolite of folic acid, is a drug widely used in the treatment of different types of cancer. When high doses are administered, it is necessary to interrupt its action by administering calcium leucovorin (CaL). The main pathway of MTX and CaL elimination in humans occurs through the kidney, but about 10% is excreted in the faeces via the bile. Drugs, foods and sorbents in intestinal lumen modify MTX and CaL reabsorption. Individual and simultaneous studies on the adsorption of MTX and CaL from aqueous phosphate buffer by cholestyramine were carried out in order to calculate the adsorption process of MTX and CaL to cholestyramine, and to characterize the influence of CaL in the adsorption of MTX to cholestyramine and vice versa. The Langmuir binding isotherms determined in buffer solutions at pH 6 indicated a greater (12.58%) adsorption capacity of cholestyramine (1.43 mmol of drug/g of resin) than at pH 7 (1.25 mmol of drug/g of cholestyramine). The affinity constant of MTX to cholestyramine was a 45.27% higher (6.67 mM^{-1}) than the affinity constant of CaL to the resin (3.65 mM^{-1}). Results from simultaneous assays indicate that a displacement of the MTX bound to cholestyramine by CaL is not foreseeable. The results suggest that cholestyramine may be a potentially useful adjunctive therapy in the treatment of an overdose of MTX. Consequently, cholestyramine may be of clinical value in patients who develop early renal function impairment whilst undergoing MTX therapy.

Keywords: Methotrexate; Calcium leucovorin; Cholestyramine; Langmuir adsorption

1. Introduction

Clinical experience with methotrexate (MTX), an antimetabolite of folic acid, is wide and its response well-known in the treatment of different types of cancer (Crom, 1998). When high doses (more than 1 g/m^2) of MTX are administered, it is necessary to interrupt its action administering calcium leucovorin (CaL), as a rescue factor, in order to prevent the haematopoietic and reticuloendothelial toxic effects. In situations of potential toxicity caused by MTX, it is necessary to increase time and the daily dose of CaL (Monjanel-Mouterde et al., 2002; van den Bongard et al., 2001).

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The major route of MTX elimination is the renal excretion. Depending on the route of administration and the length of time over which MTX is administered, the fraction of the total dose excreted unchanged in the urine can range from about 60 to 90% (Campbell et al., 1985; Webber et al., 1996; Pronzato et al., 1995). A small fraction of the administered dose may be eliminated by biliary secretion (Strum and Liem, 1977). Although this route generally accounts for less than 10% of an administered dose for high dose regimens, this may represent a significant total amount of drug (Evans et al., 1981; Crom, 1998; Creaven et al., 1973), because the number of enterohepatic cycles per day is more than one (Yamaoka et al., 1990). Therefore, this route may have an important impact on overall plasma elimination of MTX. Decreased gastrointestinal tract motility due to current drugs (vinca alkaloids, narcotic analgesics, or other anticholinergic agents) or tumour obstruction, altered renal function and/or patients poorly hydrated can produce enhanced enterohepatic recycling and prolonged exposure to toxic plasma concentrations of MTX. Usually intravenous CaL is administered after MTX administration (12 or 24 h). Even if CaL is administered by oral or intravenous its presence in the small intestine is guarantied because it is excreted via the bile and undergoes enterohepatic circulation (Hillman, 1986; Steinberg et al., 1979; Martindale, 1999).

When cholestyramine is administered by the oral route protects, total or partially, the gastrointestinal tract from the direct harmful action of MTX, but the presence of the adsorbent implies the adsorption of drugs on the resin in the small intestine, preventing their reabsorption in a quantity that not has been established yet (Erttman and Landbeck, 1985; Pérez et al., 1996; Honda and Nakano, 2000). Consequently, the clinical outcome of the simultaneous presence in the intestinal lumen of cholestyramine, MTX and CaL is unknown, and it depends on the different affinity of these drugs for this resin. If cholestyramine affinity is greater for CaL than for MTX, the latter could be reabsorbed from the intestinal tract increasing its amount in systemic circulation. In this hypothesis, administration of cholestyramine would be ineffective to get the objective to increase the total clearance of MTX. Moreover, when cholestyramine binds to CaL the elimination of this drug increases and the potential risk of toxicity caused by MTX is greater than before to administrate cholestyramine to the patient. On the contrary, if cholestyramine displayed a greater affinity for MTX than for CaL, cholestyramine could be administered without damaging CaL effectiveness and, possibly, the administration of the resin diminishing the global risk of toxicity due to MTX. Thus, when patients are in situations of toxicity, higher affinity of CaL for the resin would worse condition; on the contrary, higher affinity of MTX would be suitable, since it would not return to the systemic circulation. Therefore, it could be convenient to determine the effect of the adsorbent on the drugs in order to modify the dose of CaL administered if the loss of the drug by adsorption is significant.

The aims of this paper are to calculate the adsorption process of MTX and CaL by cholestyramine, and to characterize the influence of CaL in the adsorption of MTX to cholestyramine and vice versa. Both are preclinical aspects that will contribute to design the optimal dosage schedule for cholestyramine therapy in patients with toxicity (potential or real) by MTX, by means of suitable clinical trials.

2. Materials and methods

2.1. Substances assayed

MTX, commercialised in form of sodium salt under the registered name of Metotrexato Lederle® was used. Each vial contains 500 mg of MTX in 20 ml of solvent, with purity between 100 and 105% (lots J-13 and J-16). The vials were stored at room temperature and protected from light. The CaL used was that commercialised under the registered name of Isovorin® 175 mg, with purity between 98.8 and 103% (lots K-02 and K-03). Each vial was reconstituted with 17.5 ml of sterile water for injection, with the purpose of obtaining a final concentration of CaL of 10 mg/ml. Once the vial was reconstituted, it was stored between 2 and 8 °C and protected from light. Dissolutions were not used after 30 days of preparation. Cholestyramine resin (a bile acid sequestrant antilipemic agent) under the registered name of Resincolestiramina® was employed, with purity of 86.58% (lot J-31). It was stored in airtight containers at room temperature. All other reagents used were of analytical grade, and were employed without further purification.

2.2. Analytical procedures

Samples were assayed for MTX and CaL content by high performance liquid chromatography. The equipment consisted of a Perkin–Elmer Model 250 binary pump, a variable volume injector (ISS 200), and a Perkin–Elmer LC-90 spectophotometric detector (λ = 303 nm). A 150 mm × 4.6 mm Spherisorb S-5 ODS-2 analytical column (Phase Separations, Ltd., Queensferry) in conjunction with a C-130 B precolumn (Tecknocroma C-18) was used. The mobile phase was a mixture of acetonitrile and aqueous phosphoric acid solution 0.05 M (pH 2.7) 16:84 (v/v), at a flow rate of 1 ml/min. A 50-μl of the sample were injected into the chromatograph.

Calibration curves covering the entire range of MTX and CaL concentrations in samples were performed in triplicate. Excellent linear plots relating the peak area and solute concentration were found, and the intercept did not significantly differ from zero. Due to the simplicity of the procedure, no internal standard was needed.

The accuracy and precision of the method were validated. The criteria were assessed using four concentrations for each initial perfusion solution, covering the entire calibration range of the analytical method. Accuracy was evaluated by calculating the relative error, which was always less than 7%. Precision was evaluated by calculating the coefficient of variation, which was in all cases lower than 2%. These results were considered completely acceptable (Karnes and March, 1993).

2.3. Individual adsorption experiments

An aliquot of 10 ml of the phosphate buffer solution at 0.025, 0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml (0.055, 0.550, 1.1, 2.2, 3.3, 4.4 and 5.5 mM) of MTX or CaL, buffered to pH 6 and 7 in MTX experiments and pH 6 in CaL experiments, were added to a 25 ml centrifuge tube containing 20 mg of the Resincolestiramina®. The tubes were shaken in a thermostatically controlled water bath at 37(±0.5) °C at a speed of 70 rpm for the required equilibration time (60 min). A control without adsorbent was also carried out for each experiment to check for any change in drug stability.

The suspensions were centrifuged for 15 min at 10,000 rpm, final volume was measured and an aliquot was analysed for MTX or CaL contents. Five experiments for each initial condition were carried out.

2.4. Simultaneous adsorption experiments

In order to evaluate the interaction between MTX and CaL simultaneous adsorption experiments were carried out. In this case, 10 ml of a 0.25 mg/ml (0.550 mM) MTX phosphate buffer solution, in the presence of 0.025, 0.25, 0.5, 1.5 or 2.5 mg/ml (0.055, 0.550, 1.1, 3.3 or 5.5 mM) of CaL, buffered to pH 6, were added to a 25 ml centrifuge tube containing 20 mg of Resincolestiramina®.

The contrary process was also studied, that is, the effect of MTX on the CaL adsorption process. In this case, the amount of Resincolestiramina® (20 mg) and CaL concentration (0.25 mg/ml) were fixed, the range of MTX concentrations being 0.025, 0.25, 0.5, 1.5 or 2.5 mg/ml (0.055, 0.550, 1.1, 3.3 or 5.5 mM).

Concentrations of both drugs were selected to cover the range of possible concentrations in the small intestine in different moments of the disposition phase of the drugs (Crom, 1998).

The tubes were shaken in a thermostatically controlled water bath, and centrifuged as previously described. A subsample of the supernatant was quantified for MTX and CaL content. These assays were carried out in triplicate.

2.5. Fitting of models to data and statistical procedures

2.5.1. Remaining and adsorbed amount of the drug

A 50-μl of a standard drug dissolution and an experimental drug sample were injected into the chromatograph and the area peaks were obtained. Remaining concentration of the drug, C_r, was multiplied by the volume measured at the end of the experiment, obtaining the amount of the drug non-adsorbed by cholestyramine, Q_r.

Amounts of the drug adsorbed per unit of weight of adsorbent (mmol/g) were determined using the following equation:

$$Q_{ads} = \frac{C_i V_i - C_r V_r}{Q_{col}} \quad (1)$$

where Q_{ads} is the amount of drug adsorbed per unit of weight of adsorbent (mmol/g); C_i and C_r, initial

and final concentrations of drug in the incubation fluid (mM); V_i and V_r, initial and final volumes of the fluid (L); and Q_{col}, amount of cholestyramine used in the experiment (g).

2.5.2. Fitting of models to data

In order to characterize adsorption parameters of MTX and CaL to the resin, adsorption data were assayed to fit Langmuir isotherm expressed by the equation (Vermeulen et al., 1993):

$$Q_{ads} = \frac{Q_m K C_r}{1 + K C_r} \quad (2)$$

where Q_{ads} is the amount of the drug adsorbed per gram of adsorbent (mmol/g); C_r, concentration of unadsorbed drug at equilibrium (mM); Q_m, maximal adsorbing capacity of the adsorbent for the drug studied (mmol/g); and K, a constant indicating the affinity of the drug to the resin and physically represents the reciprocal of the free drug concentration when half of the maximal binding capacity is used (mM^{-1}).

Calculations were performed globally for all sets of data obtained in individual adsorption studies by means of three output functions (MTX pH 6 and 7 and CaL pH 6), using all individual values (naive pooled data). These fits were performed using PCNONLIN 3.0 program (Metzler, 1989). Sum of squares of residuals and standard errors of the parameters were used to assess the goodness of the fits.

Adsorption parameters of MTX and CaL to cholestyramine in the simultaneous tests were also calculated. For this, the Langmuir equation for multivariant systems (Vermeulen et al., 1993), in the particular case of two solutes binding to the same adsorbent was fitted:

$$Q_{ads} = \frac{Q_{mS} K_S C_S}{1 + C_I K_I + K_S C_S} \quad (3)$$

Q_{ads} is the amount of substrate drug adsorbed per unit of weight of adsorbent (mmol/g); Q_{mS}, capacity of the adsorbent for the substrate; C_S, concentration of unadsorbed substrate at equilibrium; K_S, affinity constant of the substrate; C_I, concentration of unabsorbed inhibitor drug; and K_I, affinity constant of the inhibitor. Experimental data obtained in single and simultaneous adsorption tests, in which the initial substrate drug concentration remains constant (0.25 mg/ml) and the initial inhibitor drug concentration is variable (0.025–2.5 mg/ml) were used.

Calculations were performed globally for all sets of data obtained by means of five output functions (MTX pH 6 and 7 and CaL pH 6, from individual adsorption studies, and two sets of data from simultaneous adsorption studies) using all individual values (naive pooled data). These fits were performed using PCNONLIN 3.0 program (simplex algorithm) (Metzler, 1989). Sum of squares of residuals and standard errors of the parameters were used to assess the goodness of the fits.

3. Results and discussion

3.1. Individual adsorption experiments

Adsorption assays have been performed at pH 6 and 7 because similar pH values have been reported for the human small intestine (Gruber et al., 1987). Initial drug concentration C_i, and percentages of drug adsorbed onto cholestyramine in each condition are shown in Table 1 for MTX at pH 6 and 7 and for CaL at pH 7 (mean values, $n = 5$, and confidence interval, 95% CI). Statistical results obtained using non-parametric Kruskal–Wallis test, performed with the percentages of drug adsorbed at each initial concentration, are also shown in table. The percentage of MTX adsorbed is always higher than 90%, in the experiments at pH 6 and 7, when the initial concentration of MTX is lower than 1 mg/ml (0.025, 0.25 and 0.5 mg/ml). These results indicate that the totality of MTX is adsorbed onto cholestyramine when the ratio drug:resin is equal or higher than 0.25, that is, a solution of 0.5 mg/ml of drug is cleaned using a suspension of 2 mg/ml of Resincolestiramina®. The results obtained indicate that when concentrations of MTX near the saturation of the resin are used the adsorbing capacity at pH 6 is higher than at pH 7.

To describe the different kinds of adsorption processes several mathematical approaches have been developed. Preliminary fits were performed using Freundlich, Langmuir and Brunauer, Emmett and Teller (BET) isotherms, and the best results were obtained when Langmuir isotherm was used. This model incorporates assumptions of monolayer adsorption and uniformity of adsorption sites. Thus, MTX and CaL

Table 1
Average and confidence interval (95% CI) of percentage adsorption values at different initial drug concentrations (C_i)

C_i (mg/ml)	Individual adsorption experiments		
	MTX		CaL
	pH 6 % adsorption (95% CI)	pH 7 % adsorption (95% CI)	pH 7 % adsorption (95% CI)
0.025	98.08 (97.35–98.81)	98.13 (97.66–98.61)	97.44 (96.17–98.70)
0.25	95.54 (93.78–97.29)	95.36 (94.54–96.18)	92.97 (90.53–95.41)
0.5	91.76 (89.84–93.67)	91.06 (88.94–93.10)	91.49 (90.29–92.70)
1.0	85.87 (81.90–89.83)	80.94 (78.28–83.60)	74.95 (70.11–79.78)
1.5	71.64 (65.97–77.31)	62.34 (53.81–70.87)	65.86 (64.39–67.34)
2.0	60.40 (54.55–66.25)	52.00 (45.47–58.53)	60.53 (57.21 –63.84)
2.5	51.76 (49.30–54.22)	47.46 (44.43–50.50)	53.19 (47.56–58.81)
χ^2	33.07	32.48	32.59
P	<0.001	<0.001	<0.001

In individual adsorption assays performed at pH 6 and 7 for MTX, and pH 7 for CaL ($n = 5$). Results obtained in the non-parametric Kruskal–Wallis test (χ^2) are also shown.

adsorption onto cholestyramine is carried out in a gradual way while the concentration of the drugs increases, until reaching a maximum adsorption (capacity constant), which is obtained when a monolayer of adsorption is formed (Cooney, 1995; Burke et al., 1991). The best model is obtained when it is considered that the capacity of the adsorbent, Q_m, is dependent on the pH of the medium, and the affinity constant, K, is a characteristic for each drug studied. Table 2 gives parameter values (Q_m and K) found for Langmuir adsorption isotherm equation obtained in the selected model and statistical figures (standard error the sum of squares of residuals and Akaike criterium) (Akaike, 1986). In Fig. 1 graphs are plotted according to the Eq. (2).

The maximal adsorbing capacity of the resin depends on the pH of the medium, which modifies the ionisation grade of the adsorbent. In fact, at pH 6 a higher number of ionised molecules of cholestyramine exist (cholestyramine is a basic compound) therefore, the capacity of the resin for ionic exchange increases. The ionisation grade of MTX is high at the two pHs assayed (99 and 94% ionised form at pH 7 and 6, respectively). This high percentage of ionisation in both conditions of pH is due to the presence of an acid carboxyl group in the MTX molecule ($pK_{a1} = 4.8$), which facilitates the ionic exchange with the resin. The degree of ionisation of the other carboxylic group present in the MTX molecule ($pK_{a2} = 5.5$, ionised 76% at pH 6 and 97% at pH 7) does not seem to affect the degree of union to the resin. This group, near the pteridin ring, presents a certain esteric impediment that makes its binding to the resin difficult.

The percentage of CaL ionised at pH 6 is also elevated (99%), the maximal adsorbing capacity to the

Table 2
Fitting parameters (mean value and standard error) of the Langmuir adsorption equation and Langmuir multivariate equation

Parameter	Individual assays (Langmuir equation)			Simultaneous assays (Langmuir multivariate equation)		
	MTX (pH 6)	MTX (pH 7)	CaL (pH 6)	MTX (pH 6)	MTX (pH 7)	CaL (pH 6)
Q_m (mmol/g)	1.434 (0.027)	1.251 (0.044)	1.434 (0.027)	1.433 (0.022)	1.250 (0.038)	1.433 (0.022)
K (mM^{-1})	6.670 (0.579)	6.670 (0.579)	3.649 (0.816)	6.690 (0.497)	6.690 (0.497)	4.202 (0.676)
SS	1.044	1.044	1.044	1.348	1.348	1.348
AIC	12.521	12.521	12.521	59.305	59.305	59.305

Obtained in the global fitting of the amounts of MTX and CaL adsorbed to cholestyramine in the individual assays and in global fitting of the amounts of MTX and CaL adsorbed to cholestyramine in the independent and combined assays. Statistical parameters, sum of squared residuals (SS) and Akaike information criteria value (AIC) are also shown.

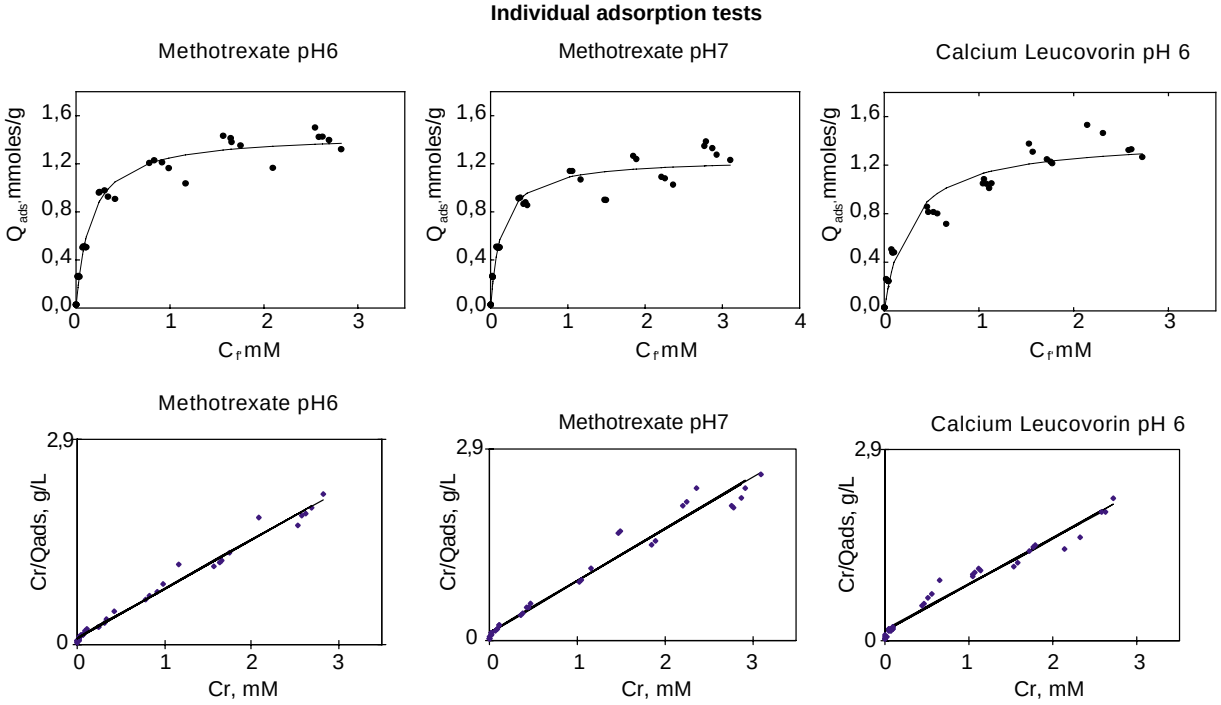

Fig. 1. Non linear (up) and linear (down) regression plot of the Langmuir adsorption isotherms for MTX and CaL. Data from individual adsorption assays. Lines represent the fit of Langmuir equation to data.

resin being equal to that obtained for MTX at pH 6 ($Q_m = 1.434$ mmol/g).

Bailey et al. (1992) obtained higher adsorption of tricyclic antidepressants (basic compounds) onto cholestyramine when the pH was reduced from 4 to 1, that is, when both adsorbent and adsorbate were in their ionised forms. Nevertheless, the authors also observed an increase in the adsorbing capacity when pH increased from 4 to 6.5, although the ionisation grade for both adsorbent and adsorbate decreased. They suggested the existence of two pathways of adsorption of drugs onto cholestyramine, one of which is due to a non-electrostatic mechanism between adsorbate and hydrophobic sites of the adsorbent. This behaviour has also been postulated by other authors in adsorption studies of bile acids onto cholestyramine (Honda and Nakano, 2000; Johns and Bates, 1969).

Values of the maximal adsorbing capacity of the resin for MTX reported in this study differ from those reported by other authors (Honda and Nakano, 2000), who indicated a capacity factor for cholestyramine equal to 1.804 mmol/g at both pHs. These differences might be due to the solvent or to the fact that a different kind of cholestyramine has been used. Results reported by Honda and Nakano (2000), were obtained using pure cholestyramine in water, and our experiments were carried using Resincolestiramina® with a purity of 86.58% ($1.804 \times 0.8658 = 1.562$ mmol/g) and phosphate buffer at pH 6 or 7 as solvent.

The constant of MTX indicating the affinity of the drug to cholestyramine, $K_{MTX} = 6.67\,mM^{-1}$, is higher (45.27%) than that of CaL, $K_{CaL} = 3.65\,mM^{-1}$ (Table 2). Moreover, the product of K and Q_m was calculated because this parameter is an "alternative" indicator for a drug affinity. The values obtained (9.564 for MTX, pH 6; 8.344 for MTX, pH 7, and 5.233 for CaL, pH 6) corroborate that MTX pH 6 has higher affinity to the resin. This fact can be attributed to the molecular structure of the two drugs. The presence of some chemical groups in the chemical structure can vary the adsorption processes. Some authors have reported that when the adsorbate is provided of hydroxyl or amino groups adsorption to charcoal is reduced. Johns and Bates (1969), using cholestyramine

demonstrated that the affinity constant decreases when a hydroxyl group is added to the bile salt structure. These results suggest that non-electrostatic forces between hydrophobic sites of the adsorbent and adsorbate exist. The presence of an additional hydroxyl group in the CaL molecule could explain the lower affinity to cholestyramine found in our conditions.

3.2. Simultaneous adsorption experiments

Percentage of MTX and CaL adsorbed at each initial concentration of CaL and MTX, respectively, obtained in simultaneous adsorption assays are given in Fig. 2. The percentage of adsorption of MTX is higher than that of CaL in all conditions assayed. Fig. 2 shows that 1.5 mg/ml of CaL (weight relation MTX/CaL 1/6) is needed to reduce the percentage of MTX adsorbed onto cholestyramine below 90%. Also, presence of MTX modifies the adsorption of CaL onto cholestyramine. An initial concentration of MTX of 0.25 mg/ml is needed (CaL/MTX ratio 1:1) in order to obtain percentages of CaL adsorbed below 90%.

Langmuir multivariate equation was fitted to the data found in individual and simultaneous adsorption tests. Parameter values and statistical figures obtained after fitting the multivariate Langmuir equation to the data (five sets, results from individual and simultaneous assays) are shown in Table 2. As can be seen, results obtained for MTX and CaL in individual adsorption tests are similar to those acquired when both experimental data from individual and simultaneous adsorption sets are used. These results are in agreement with the percentages of adsorption found in the simultaneous tests, which were superior for MTX, and with the results obtained from individual adsorption of MTX and CaL tests, in which the affinity constant of MTX was 1.82-times greater than that of CaL. Therefore, considering the value of the affinity constants, in both independent and simultaneous adsorption tests, it is possible to affirm that cholestyramine fixes selectively to MTX rather than to CaL.

Assays performed in rats by Pérez et al. (1996) demonstrated that plasma concentration of MTX 4 h post-perfusion was reduced a 49% when cholestyramine was administered by the oral route. This reduction was attributed to adsorption of drug in intestinal lumen onto cholestyramine increasing its elimination into faeces. Other experiments carried out in rats indicated that total clearance of MTX increases a 27% and area under the curve decreases a 26.64% when

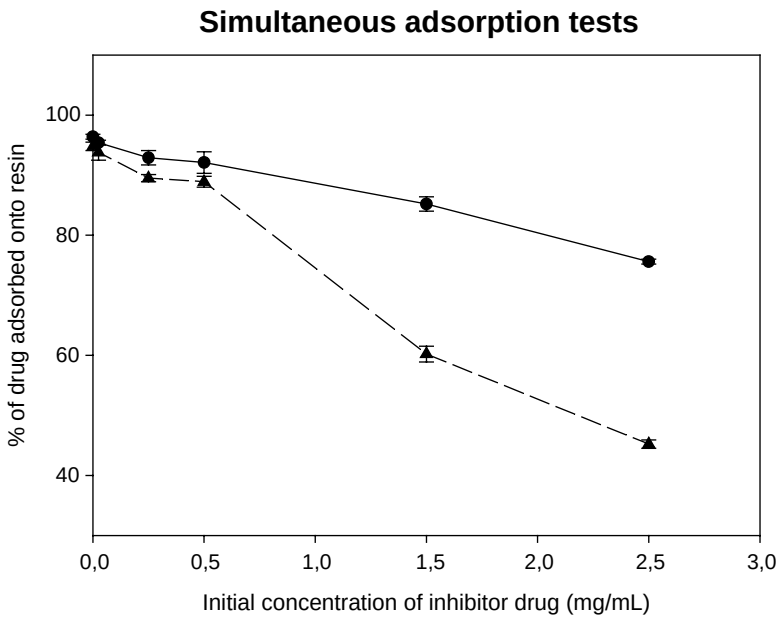

Fig. 2. Percentage of MTX (●) and CaL (▲) adsorbed onto cholestyramine vs. initial concentration of CaL and MTX, respectively, of the simultaneous adsorption tests. The standard error of the mean value is also represented.

14 mg/kg of MTX are administered by i.v. perfusion and 50 mg of cholestyramine are administered orally before perfusion and 2, 4, 6, 18 and 30 h post-perfusion (Ferrer-Bosch, 2001). In these conditions a 21.49% of the administered dose is adsorbed to the resin, that is, 2.83 mg of MTX/g of cholestyramine administered. The value of the maximal capacity of the resin obtained in our study varies from 649 to 567 mg of drug/g indicating that in vivo conditions are far from the saturation of the resin.

4. Conclusion

The usefulness of oral cholestyramine to decrease MTX toxicity by inhibiting its intestinal reabsorption depends on the ratio of concentrations of MTX, CaL and cholestyramine in the luminal content. Keeping in mind that MTX and CaL are excreted via the bile, it is necessary that when cholestyramine is administered orally to reach the small intestine, CaL concentration always has to be equal or inferior to MTX concentration in order to decrease the reabsorption of MTX, preventing its toxicity and avoiding in this way CaL elimination. From the results obtained in this study, it can be concluded that a displacement of the association of MTX to cholestyramine by CaL is not foreseeable. Consequently, it could be possible to use cholestyramine by the oral route in order to prevent reabsorption of MTX excreted via the bile, thus improving the elimination of the drug in the faeces.

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