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Inhibitory effect of bile salts on the enterohepatic circulation of methotrexate in the unanesthetized rat: Inhibition of methotrexate intestinal absorption*,**

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Summary. The effect of conjugated and unconjugated bile salts on the intestinal absorption of methotrexate (MTX) in the unanesthetized rat was investigated using a recycling perfusion technique. We initially determined the general characteristics of MTX absorption in vivo. Absorption of low (0.5 μM) and high (6 μM) concentrations of MTX was linear with time for 60 min perfusion and occurred at rates of 0.2 and 1.65 nmol/100 cm dry length/min, respectively. Absorption of 0.5 μM MTX was pH-dependent and increased with decreasing perfusate pH. Absorption of MTX involves two processes: (1) a saturable process with a K_t of 0.98 μM, and (2) a nonsaturable diffusion process. The unconjugated deoxycholate and the conjugated taurocholate inhibited the intestinal absorption of 1 μM MTX in a concentration-dependent manner. The inhibitory effect of bile salts was reversible, and was not due to damage to the intestinal mucosa. The structural analogues folic acid and 5-methyltetrahydrofolate and the organic anions rose bengal and sulfobormophthalein were also inhibitory to MTX absorption. This study demonstrates that a variety of organic anions inhibit MTX intestinal absorption. The possible therapeutic importance of this observation is discussed.

Introduction

The folate antagonist MTX is a potent inhibitor of the enzyme dihydrofolate reductase and has been shown to be effective in the treatment of various malignancies [1, 6, 11]. A major limiting factor in the use of MTX is its toxicity toward nonmalignant, rapidly proliferating tissue, especially that of the small intestine [1]. The toxicity may be potentiated by prolonged retention of MTX in the body because of the enterohepatic circulation [1, 11, 12, 15].

Using in vitro tissue preparations, we have recently shown that unconjugated deoxycholate and cholate at nontoxic concentrations interrupt MTX enterohepatic circulation by inhibiting its intestinal transport, liver uptake, and biliary secretion [10]. In vitro observations, however, do not necessarily reflect the in vivo situation. Bile salts have been shown to inhibit the intestinal absorption of alanine in vitro but not in vivo [3]. Due to these facts and our interest in developing method(s) for decreasing or eliminating MTX intestinal toxicity [10] we extended our previous in vitro observations to the in vivo system in the unanesthetized restrained rat and further examined the effect of conjugated taurocholate, structural analogues, and organic anions on the intestinal absorption of MTX. We also determined the general characteristics of the MTX intestinal absorption process in vivo.

Materials and methods

Chemicals. The following materials were obtained commercially: unlabeled MTX, folic acid, 5-methyltetrahydrofolate, deoxycholic acid-sodium salt, taurocholic acid-sodium salt, rose bengal, and sulfobromophthalein from Sigma Chemical Company; [3',5',7-^{3}H] MTX-sodium salt (238 mCi/mmol) from Amersham/Searle Corporation, Des Plaines, Ill; scintillation fluid, Ultrafluor and the one-step digestant and scintillator, Fluorosol, from National Diagnostics, Sommerville, NJ. All chemicals were of analytic quality. The radioactively labeled MTX was purified before use on cellulose-precoated thin-layer chromatography plates (Eastman Kodak Company, Rochester, NY), 160 mM phosphate buffer at pH 7.0 being used as the solvent system [4]. The purified ^{3}H-MTX used in this study was 98% radiochemically pure.

Absorption study. Unfasted male Sprague-Dawley rats weighing 180–250 g were anesthetized with ether, and the abdomen was opened by a midline incision. A polyethylene inflow catheter was inserted into the proximal end of the jejunum, 14 cm distal to the pylorus. An L-shaped glass outflow cannula was then introduced 30 cm distal to the inflow catheter. The catheters were secured by encircling ligatures which passed between the parallel end-vessels without obstructing blood vessels or lymphatics. The intestinal segment was flushed with phosphate buffer to remove any residual intestinal contents, and then flushed with air to minimize the amount of residual fluid left in the segment. The intestinal segment was then placed in the peritoneal cavity and the abdomen was closed. The animal was allowed to awaken and was placed in a restraining cage. The inflow cannula to the intestinal segment was then connected to a reservoir containing 30 ml perfusate. The perfusate used was Krebs-Ringer

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phosphate buffer and contained: 20 m*M* NaH_2PO_4, 125 m*M* NaCl, 4.43 m*M* KCl, 1.23 m*M* $MgSO_4$, 0.85 m*M* $CaCl_2$, and 10 m*M* glucose. The pH of the perfusate, unless otherwise mentioned, was adjusted to 6 with 1 *M* NaOH. A totally occlusive roller pump (Buchler Instruments Inc., Fort Lee, NJ) was used to pump the perfusate (4 ml/min) from the reservoir through the inflow catheter and into the intestinal segment. The outflow cannula was allowed to drain back into the reservoir by gravity. The solution in the reservoir was stirred continuously with a magnetic stirrer. A forced air heating device and a thermostatic temperature controller (Thermistemp Model 74, Yellow Spring Instruments, Yellow Spring, Ohio) were used to monitor the animal's body temperature with a rectal probe and maintain the animal's temperature at 37 °C. The rate of MTX absorption was calculated by determining its rate of disappearance from the perfusate. Fluid shifts which might take place during the perfusion were assessed with the use of ^{14}C-labeled inulin as a nonabsorbable marker and the absorption rate of MTX was corrected for fluid shifts. ^{3}H- and ^{14}C-radioactivity were determined by a double isotope counting and calculating technique [7]. Perfusion was performed for 1 h. Aliquots (100 µl) in quadruplicate were removed from the reservoir at the end of perfusion, added to scintillation vials containing 6 ml scintillation fluid, and subsequently analyzed for radioactivity. At the end of the experiment, each animal was killed by an overdose of ether and the perfused intestinal segment was removed and washed with 30 ml phosphate buffer. A 10-g weight was attached to the most dependent portion of the segment to insure a constant degree of bowel stretch during drying. After a 24-h drying period at room temperature the length of the segment was measured and recorded. The absorption results were expressed in terms of dry intestinal length, which correlates best with the intestinal surface area [17].

In preliminary experiments we examined the possible adsorption of MTX to the tubings and reservoir and found that adsorption was less than 1% during 1 h of perfusion.

Method of Calculation and Statistical Analysis. The amount of MTX absorbed after each time period was calculated from the following equation:

$$A = B\left\{1 - \frac{^{14}C_o \cdot {}^{3}H_t}{^{3}H_o \cdot {}^{14}C_t}\right\}$$

where A represents the amount of the substrate absorbed (disappeared) from the perfusate in nanomoles; B represents the total amount of the substrate in nanomoles added to the perfusate at time zero; $^{14}C_o$ and $^{3}H_o$ represent the corrected counts per minute at time zero (standard) and $^{14}C_t$ and $^{3}H_t$ represent the corrected counts per minute at time t.

All the results presented in this paper are means ± SEM of at least five separate observations in at least five animals. Data were analyzed with reference to Student's *t*-test and regression analysis.

Results

Effect of time

Absorption of 0.5 and 6 µ*M* MTX as a function of time was examined to determine the linearity of the absorption process. Perfusion experiments were performed for 60 min, and sampling in duplicate was performed at 10-min intervals. After an initial rapid disappearance of the substrate from the perfusate, which is represented by the intersection of the lines with the *y*-axis and which is probably due to a rapid binding to the mucosal surface, absorption of 0.5 and 6 µ*M* MTX increased linearly with time for 60 min (Fig. 1). The rates of absorption of 0.5 and 6 µ*M* MTX were calculated by linear regression analysis and were found to be 0.20 ± 0.03 and 1.66 ± 0.10 nmol/-100 cm dry length/min, respectively.

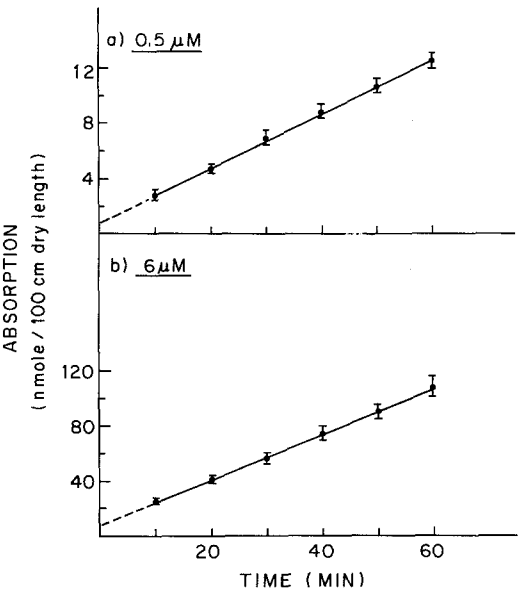

Fig. 1 a, b. Time course of MTX absorption from the lumen of rat jejunum in vivo. Initial concentrations of MTX were 0.5 µ*M* **(a)** and **b** 6 µ*M*. Linearity and rate of absoprtion as a function of time were calculated by means of regression analysis ($Y = mX + b$; $r = 0.99$ and 0.99, $m = 0.2$ and 1.66 and $b = 0.132$ and 7.79 for 0.5 and 6 µ*M* MTX, respectively). Each *data point* represents the mean ± SEM of at least five observations in five rats

Effect of pH

The absorption of 0.5 µ*M* MTX was examined at perfusate pH 7.5, 6.0 and 5.0 (Table 1). The amount of MTX absorbed after 60 min perfusion increased as the pH of the perfusate decreased. Changes in perfusate pH were observed at the end of perfusion when the pH was 7.5 or 5.0, but not when the pH was 6.0. We decided to use pH 6.0 in all our experiments because of its stability and its good approximation to the physiological pH [9].

Effect of concentration

The rate of absorption (nmol/100 cm dry length/min) of MTX as a function of increasing the drug concentration in the perfusate was examined at pH 6.0 over a range of concentrations from 0.1 to 50 µM (Fig. 2). Saturation in the absorption process was observed at low concentrations of MTX (< 10 µ*M*). At higher concentrations (> 10 µ*M*), the absorption was linear. To eliminate the contribution of the linear (diffusion) process in the absorption of low concentrations of MTX, we determined the diffusion rate (slope) at high concentrations of MTX (20–50 µ*M*), then multiplied the diffusion rate (0.213 nmol/100 cm/min) by the individual concentration. We then substracted the diffusion

Table 1. Effect of perfusate pH on the absorption of 0.5 μ*M* MTX in 50-cm jejunal segments

Perfusate pH		Absorption (nmol/100 cm/h)	*P* Value[a]
Initial	Final		
7.5 ±0.2	7.0 ±0.05	9.7 ±0.5	
6.0 ±0.02	6.0 ±0.03	12.4 ±0.1	<0.01
5.0 ±0.01	5.6 ±0.03	13.5 ±0.6	<0.01

[a] Comparison against amount of MTX absorbed at pH significance of differences determined according to 7.4; Student's *t*-test

process from the total absorption at each concentration. K_t and V_{max} of the saturable process were then determined by means of a Lineweaver-Burk plot (insert, Fig. 2) and regression analysis, and found to be 0.98 μM and 0.36 nmol/-100 cm/min, repectively.

Effect of bile salts, structural analogues and organic anions

In this study we examined the effect of deoxycholate, the conjugated taurocholate, the structural analogues folic acid and 5-methyltetrahydrofolate, and the organic anions rose bengal and sulfobromophthalein on the absorption of 1 μ*M* MTX in vivo (Table 2). Both deoxycholate and taurocholate inhibited the intestinal absorption of MTX in a concentration-dependent manner. Tissue histology, which was examined in specimens taken from the middle portion of the perfused jejunal segments at the end of the perfusion and stained with PAS [8], showed intact intestinal villi and epithelial cells in both untreated and bile-salt-treated tissue. The reversibility of the inhibitory effect of the bile salts on the intestinal absorption of MTX was also examined. In this experiment we preperfused 30-cm jejunal segments with phosphate buffer containing 0.5 m*M* deoxycholate or 10 m*M* taurocholate, flushed the intestine with 60 ml phosphate buffer to wash out the bile salt, then flushed the segment with air to minimize residual fluid. We then performed absorption studies for 60 min in the presence of 1 μ*M* MTX in the perfusate. Control experiments were performed in a similar way, except that the bile salt under investigation was not added to the perfusate. The results showed no inhibition in the absorption of MTX following preperfusion with bile salt (26.0±2, 24.0±1.9 and 25.1+3 nmol/
100 cm dry length/h of MTX was absorbed in control intestine and in intestine treated with 0.5 m*M* deoxycholate and 10 m*M* taurocholate, respectively).

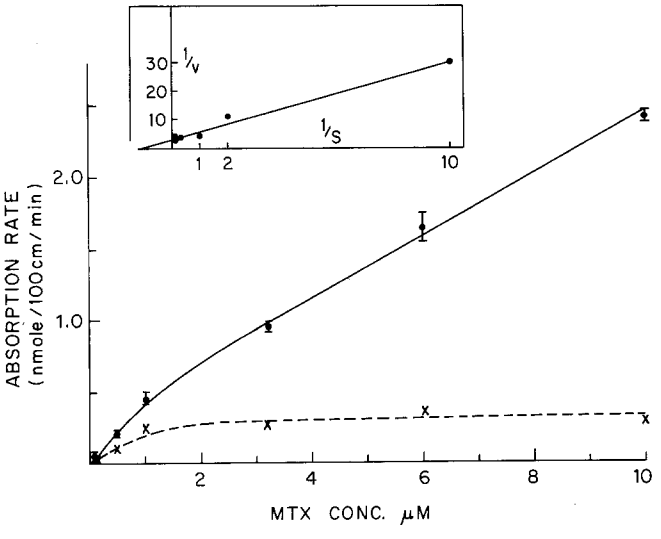

Fig. 2. Effect of concentration on the rate of absorption of MTX. The *solid line* represents total absorption (saturable plus diffusion processes) and the *dashed line*, the saturable process only. K_t and V_{max} of the saturable process were calculated from the Lineweaver-Burk plot by means of linear regression analysis ($Y=mX+b$; $r=0.99$, $m=2.74$ and $b=2.79$). Each *data point* represents the mean ±SEM of at least five observations in five rats

Table 2. Effect of bile salts, structural analogues and organic anions on the absorption of 1 μ*M* MTX in 50-cm jejunal segments

Compound	Absorption (nmol/100 cm/h)	*P* Value[a]
Control	30.4 ±0.8	
Deoxycholate		
0.01 m*M*	24.6 ±0.7	<0.01
0.1 m*M*	21.8 ±1.0	<0.01
0.5 m*M*	11.6 ±0.5	<0.01
Taurocholate		
0.1 m*M*	28.7 ±2.2	NS
1.0 m*M*	21.9 ±1.5	<0.01
10 m*M*	15.6 ±0.7	<0.01
Folic acid		
50 μ*M*	8.8 ±0.4	<0.01
5-Methyltetrohydrofolate		
50 μ*M*	9.9 ±0.4	<0.01
Sulfobromophthalein		
0.1 m*M*	14.8 ±2.4	<0.01
0.1 m*M*	9.3 ±2.0	<0.01
Rose Bengal		
0.1 m*M*	14.7 ±0.9	<0.01

[a] Comparison against control; significance of differences determined according to Student's *t*-test

Not only bile salts but also the structural analogues folic acid and 5-methyltetrahydrofolate and the organic anions sulfobromophthalein and rose bengal were inhibitory to the intestinal absorption of 1 μ*M* MTX (Table 2).

Discussion

This study was designed to examine the effect of unconjugated and conjugated bile salts on the intestinal absorption of MTX in vivo in unanesthetized restrained rats. We first determined the general characteristics of the MTX absorption process with reference to time, buffer pH, and concentration. Absorption of 0.5 and 6 μ*M* MTX was linear with time for 60 min of perfusion. Absorption of MTX was pH-dependent and increased as perfusate pH decreased. The absorption of MTX in vivo involves two processes: a saturable process, which operates at low concentrations of the compound and has a K_t of 0.92 μ*M* and V_{max} of 0.34 nmol/100 cm/min; and a nonsaturable (diffusion) process that operates at high concentrations (<10 μ*M*). These observations are in good agreement with those previously reported following in vitro studies on MTX intestinal transport in the everted sac preparation [13, 14, 16]. Both the unconjugated deoxycholate and the conjugated taurocholate inhibited the intestinal absorption of MTX.

The inhibition was reversible upon the removal of the bile salts from the perfusate and was not due to damage to the intestinal mucosa. This observation confirms our previous in vitro findings [10] on the effect of unconjugated bile salts on MTX absorption and further demonstrates that conjugated bile salts are also inhibitory. The inhibitory effect of bile salts on MTX absorption in vivo as well as in vitro is in contrast to their inhibitory effect on alanine absorption in vitro but not in the in vivo setting [3].

Not only bile salts but also the structural analogues folic acid and 5-methyltetrahydrofolate and the structurally unrelated organic anions sulfobromophthalein and rose bengal were inhibitory to MTX absorption. These observations suggest the possibility that the anionic MTX and organic anions may share the same transport system in the intestine. Similar observations and suggestions have been reported for MTX and organic anions in freshly isolated rat hepatocytes [2].

We have recently shown, by in vitro techniques, that bile salts interrupt the enterohepatic circulation of MTX by inhibiting its intestinal transport, liver uptake and bile secretion, with no damaging effect to the intestine or the liver [10]. On the basis of these observations we proposed that bile salts should be given in therapeutic doses in an attempt to decrease MTX intestinal toxicity [10]. Bile salts should not affect the interaction of MTX with its target tumor site, because (a) the extremely efficient removal of bile salts from the portal circulation by hepatocytes would remove the majority of bile salts from the general circulation on the first pass through the liver [5]; and (b) high concentrations of bile salts are required to inhibit the transport of MTX into tumor cells [4]. The present in vivo observations clearly support our previous observations and proposal.

In summary, the present study shows that a variety of organic anions interfere with the intestinal absorption of the anionic MTX in the unanesthetized rat. Studies are currently under way to examine the effect of different bile salts and organic anions on liver uptake and bile secretion of MTX in the unanesthetized rat.

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