Enterohepatic Circulation of Methotrexate in Rats in Vivo

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ABSTRACT

The pharmacokinetics of the methotrexate enterohepatic cycle were studied in rats in vivo. For plasma levels of methotrexate between $10^{-5}$ and $10^{-9}$ mM, biliary levels were directly proportional and concentrated 27-fold. When labeled methotrexate was administered in doses sufficient to achieve plasma levels of $10^{-9}$ mM, approximately 50% of methotrexate appeared in the bile in normal animals and up to 80% appeared in anephric animals. In spite of the high percentage of administered methotrexate which appeared in the bile, complete interruption of the enterohepatic cycle in otherwise normal animals did not affect the plasma decay curve of a bolus of methotrexate. The increased biliary excretion which occurred in animals with renal impairment was utilized with possible therapeutic implications. Bile drainage in these animals rapidly decreased plasma methotrexate levels compared to nondrained controls. This suggests that interruption of the methotrexate enterohepatic cycle may provide an alternative for the management of methotrexate toxicity associated with renal insufficiency.

INTRODUCTION

The pharmacokinetics of methotrexate has been under active investigation for more than 15 years (1, 6, 8, 12, 18). Most attention has been given to measurements of serum clearance, tissue uptake and turnover, binding to dihydrofolate reductase, and renal clearance. Methotrexate secretion into bile was first studied by Henderson et al. (13) in 1965. Although determinations have been made of the transport of methotrexate from plasma to bile (3, 5, 15), studies of the contribution of the enterohepatic pathway to methotrexate pharmacokinetics have been limited (5). Since methotrexate shares many properties with its folate analog methyltetrahydrofolate, we utilized a model developed previously for studies of the folate enterohepatic cycle (20) to investigate this aspect of methotrexate pharmacology.

MATERIALS AND METHODS

Female Sprague-Dawley rats weighing 175 to 250 g were used in all experiments. Methotrexate was obtained as methotrexate sodium, 25 mg/ml in 2-ml vials; folic acid (PteGlu) was obtained as Folvite, 5 mg/ml in 10-ml bottles; and leucovorin (folinic acid, citrovorum factor, CHO$_2$PteGlu) was obtained as calcium leucovorin, 3 mg/ml in 1-ml vials; all were obtained from Lederle Laboratories Division, American Cyanamid, Pearl River, N. Y. [3',5',7-3H]Methotrexate sodium salt, 10 Ci/mmol, was obtained from Amersham/Searle Corp., Arlington Heights, Ill. The isotope was shown to be greater than 98% pure when chromatographed on Sephadex DEAE-A-25 (19).

Methotrexate levels were performed by the dihydrofolate reductase inhibition assay, with Lactobacillus casei reductase obtained from New England Enzyme Center, Boston, Mass. (2). The lower limit of sensitivity of the assay was determined to be $10^{-9}$ M for methotrexate diluted in either rat plasma, bile, or urine. There was less than 10% cross-reactivity with 7-hydroxymethotrexate. Chromatographic analysis of bile and urine for methotrexate and its metabolites was carried out on 1.5- x 30-cm columns of Sephadex DEAE-A-25 (19). A linear gradient of 0.1 to 1.0 M potassium phosphate buffer, pH 6.0, containing 200 mM 2-mercaptoethanol was used for elution. Labeled methotrexate that was used as a marker eluted at volumes 325 to 375 ml.

A rat model developed previously for studies of the folate enterohepatic cycle (20) was used to study the methotrexate enterohepatic cycle and its relationship to methotrexate elimination into urine. Four aspects of methotrexate kinetics were studied.

Correlation of Plasma and Bile Methotrexate Levels. Fifteen animals were anesthetized with Innovar vet and 0.965-mm central venous catheter was placed via the jugular. Bile-drained animals had their bile duct isolated and cannulated with a 0.965-mm polyethylene catheter. Biliary flow was constant at approximately 1 ml/hr for the duration of this and subsequent experiments. The biliary catheter was tunneled to a position at the back of the neck and through a harness and metal conduit out of the cage. This permitted the animal to be awake and taking food and fluids ad libitum while bile was drained and blood samples were taken. Fifty mg methotrexate (250 mg/kg) was administered by tail vein. Bile was collected in iced, light-shielded vials. Simultaneous plasma and bile samples were obtained at intervals between 45 min and 2 hr for methotrexate assay. Sampling was delayed until 45 min to allow equilibration between compartments. Six animals were also studied after an 18-hr continuous infusion of methotrexate (see below).

Clearance of Methotrexate following Bolus Injection. Plasma methotrexate clearance following bolus injection was characterized in 6 normal and bile-drained rats. All rats were anesthetized and had central venous and biliary catheters placed as above. Fifty mg methotrexate (250 mg/kg) were injected by tail vein, and plasma samples were collected from the venous catheter serially over 48 hr.

Relationship of Biliary to Renal Methotrexate Excretion. To duplicate the clinical practice of high-dose methotrexate infusion, the dosage of methotrexate required to achieve and maintain plasma levels of approximately $10^{-9}$ in a 200-g rat was calculated. A loading methotrexate dose of 3.7 mg/kg [including [3H]methotrexate (5 μCi/143 ng)] was administered to normal animals via jugular catheter, followed by a continuous infusion of unlabeled methotrexate at 0.75 mg/kg/hr. With this regimen, the steady-state methotrexate level was 1.86 ± 0.55 (S.E.) x $10^{-9}$ M (n = 46). All urine was collected for 2 hr, after which the bladder was aspirated to ensure complete collection. The urine was then pooled and counted to determine the total amount of isotope appearing in urine in 2 hr.

As an extension of this study, groups of animals had excretion measured while either the biliary or renal excretion pathway was altered as follows. (a) On the day prior to the experiment, 6 animals had a laparotomy performed, and the bile duct was ligated. They were then...
treated in a fashion identical to that of the normal animals. (b) A group of 6 animals had both central venous and bile catheters placed before administration of methotrexate, after which all bile and urine were collected for 2 hr after administration of the isotope. (c) Six animals had a right nephrectomy 6 days before the experiment. On the day of the experiment, a cannula was placed in the bile duct, and the remaining kidney was removed. The loading dose containing [3H]methotrexate followed by a continuous infusion was again administered, and all bile was collected for 2 hr from the anephric animals.

The same series of experiments were repeated in animals subjected to an 18-hr continuous infusion of methotrexate. On the day prior to the experiment, animals had a central venous catheter implanted, which was tunneled to a position at the back of the neck and through a harness and metal conduit to a position outside of the cage. The animals were awake, eating, and drinking normally while receiving a continuous infusion of unlabeled methotrexate (0.75 mg/kg/hr after a loading dose of 3.75 mg/kg as before). They were then treated in a fashion similar to that described for the short-term infusion as follows. (a) Normal animals (animals with an intact enteric cycle) were given an i.v. bolus of 5 µCi (143 ng) [3H]methotrexate at 18 hr. All urine was collected for the next 2 hr, after which the bladder was aspirated. (b) Bile duct-ligated animals had a laparotomy performed, and their bile duct was ligated on the same day the central venous catheter was placed, before initiating the methotrexate infusion. All urine was collected for 2 hr after injection of the isotope, and the bladder was then aspirated. (c) Bile duct-ligated animals had a laparotomy performed, and the bile duct was cannulated just prior to the administration of [3H]methotrexate. Both bile and urine were then collected for 2 hr after isotope administration. (d) Anephric animals, as with the short-term infusion model, had a right nephrectomy 6 days before the experiment. Just prior to the administration of the [3H]methotrexate, a laparotomy was performed; the bile duct was cannulated, and the remaining kidney was removed. Bile was again collected for 2 hr.

Effect of Bile Drainage on Plasma Methotrexate Levels. To examine the relationship of the enteric cycle and renal function in supporting plasma methotrexate levels, normal and 2 groups of renal-impaired animals (nephrectomized and bladder excised) undergoing methotrexate infusion were subjected to bile drainage. In normals and in each group of renal-impaired animals, non-bile drained but similarly operated animals served as controls: (a) Twelve normal-renal-function animals had central venous catheters placed and methotrexate infusions begun as above. After 18 hr of infusion, all animals were anesthetized, 6 for sham laparotomy (controls) and 6 for laparotomy and placement of a bile drainage catheter (bile drained). The steady state infusion was continued for 8 hr, and plasma samples were obtained every 2 hr for methotrexate levels. (b) Two groups of renal-impaired animals undergoing methotrexate infusions were subjected to prolonged bile drainage. Eight animals underwent a right nephrectomy 6 days before the experiment. The nephrectomized animals and 8 normal animals (for bladder excision) had central venous catheters placed and methotrexate infusions begun as above. After 18 hr of infusion, the 2 groups were subjected to a laparotomy. The second kidney was removed from the nephrectomized animals. In the normal animals, the bladder was excised so that all flow from the ureters emptied into the peritoneal cavity. One-half of the animals from each group had a biliary cannula placed to drain all bile, whereas the other half served as non-bile-drained controls. The methotrexate infusions were then discontinued, since our preliminary studies had demonstrated that in the absence of renal function this would result in a slow decline in plasma methotrexate levels. Plasma samples were obtained every 2 hr for methotrexate levels.

Finally, an attempt was made to increase biliary excretion of methotrexate in anephric animals by administering high doses of folic or folinic acid. The protocol was identical to that just described except that folinic acid (2 mg) or folic acid (2 mg) was given every 20 min for the first 3 hr.

RESULTS

Studies of the methotrexate enterohepatic cycle and its relationship to urinary clearance of the drug gave the following results.

Correlation of Plasma and Bile Methotrexate Levels. The excretion of methotrexate (MTX) into bile correlated with the plasma level in a relationship described by first-order kinetics:

\[
(MTX_{\text{bile}}) = 27.4 \times (MTX_{\text{plasma}})^{0.9 \pm 0.1}
\]

\( n = 27, r = 0.96, p < 0.001 \) (Chart 1). There was a 27-fold concentration of methotrexate in bile over plasma, a relationship that held over a 2- to 3-log range in the plasma methotrexate concentration.

Clearance of Methotrexate following Bolus Injection. Chart 2 shows the plasma concentration of methotrexate over time, following bolus injections, in normal and bile-drained animals. Elimination of methotrexate recirculation through the enterohepatic cycle by bile drainage had no effect on the decline of the plasma concentration.

Relationship of Biliary to Renal Methotrexate Excretion. Urinary and biliary excretion of a tracer dose of [3H]methotrexate were compared in animals undergoing short-term and continuous infusions of unlabeled methotrexate (Table 1). Normal animals undergoing short-term methotrexate infusions excreted 35.7 ± 3.1% of the isotope in urine within 2 hr of being injected. Similarly, continuous-infusion animals excreted 38.4 ± 5.2% within 2 hr of isotope administration. In both short-term and continuous experiments, interruption of the enterohepatic cycle by bile drainage did not affect urinary excretion during the 2-hr collection period. When the bile duct was ligated on the day prior to the experiment, urinary excretion increased significantly (88.0 ± 8.5% in short-term infusion animals, p < 0.005; 65.7 ± 2.2% in continuous-infusion animals, p < 0.01).

Almost 50% of the administered methotrexate isotope was excreted into bile in the first 2 hr (short-term animals, 47.8 ±...
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4.0% and continuous-infusion animals, 55.6 ± 2.0%). Biliary excretion was even greater in anephric animals (62.1 ± 6.4% in short-term animals, p < 0.001; 67.8 ± 3.8% in continuous-infusion animals, p < 0.01).

**Effect of Bile Drainage on Plasma Methotrexate Levels.** Studies were also performed to determine the role of the methotrexate enterohepatic cycle in supporting plasma methotrexate levels in normal animals and those with impaired renal function. In normal animals, prevention of enterohepatic cycling by bile drainage for 8 hr did not alter the plasma methotrexate level (2.1 ± 0.4 × 10⁻⁶ M) compared to nondrained controlled animals (1.9 ± 0.6 × 10⁻⁶ M); that this was in part due to renal compensation was demonstrated when similar experiments were performed in renal-impaired animals (Chart 3). As expected, impaired renal function resulted in delayed plasma clearance of the drug. With the enterohepatic cycle intact, plasma methotrexate values for bladder-excised animals declined more rapidly than did those for anephric animals, suggesting that some of the methotrexate cleared into the peritoneum was not immediately reabsorbed. Bile drainage effectively decreased the plasma methotrexate level in each renal-impaired group.

Finally, administration of large doses of PteGlu, or folinic acid neither increased methotrexate secretion into the bile nor enhanced the effect of bile drainage in decreasing plasma methotrexate levels in anephric animals (data not shown).

**DISCUSSION**

Since the early work of Henderson et al. (13), models of methotrexate pharmacokinetics have included a methotrexate enterohepatic cycle (3–5, 7, 10, 11, 14, 17, 22, 23). It is also well known that the concentration of methotrexate in liver and bile exceed that of plasma, although there has been disagreement as to the correct liver:plasma or bile:plasma methotrexate ratio (3, 6, 7, 9, 15–17, 22). In this study, the ratio of bile to plasma methotrexate concentration was compared in animals receiving a bolus injection of the drug and also during continuous infusions. Following bolus injections, sampling was delayed for 40 min to permit equilibration. The concentration of methotrexate in bile correlated directly with the plasma level in a first order relationship (Chart 1, open circles) with a concentration factor of 27. This is consistent with results obtained by Kates and Tozer (15) showing that the methotrexate clearance from serum into bile was a first-order process which was saturable but with a high (7 × 10⁻⁵ M) half-saturating concentration. Animals receiving a continuous (18 hr) infusion of unlabeled methotrexate were also studied (Chart 1, solid circle). In this situation, in which there would be no chance for a mismatch due to delayed equilibration, there was also a 27-fold concentration of methotrexate in bile over blood. This value differs from various other data reported previously (3, 6, 7, 10, 18, 22) and may be related to species variations.

Studies were then performed to compare quantitatively the excretion of methotrexate in bile and urine (Table 1). In normal animals, biliary excretion of methotrexate given either during a short-term or overnight infusion surpassed renal excretion. Clearly, hepatic clearance into bile is an important route of elimination from the plasma compartment. This is further supported by the increase in urinary excretion (Table 1) and

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**Table 1**

Percentage of excretion of [³H]methotrexate in bile and/or urine during short-term and continuous (18 hr) methotrexate infusions

<table>
<thead>
<tr>
<th></th>
<th>Short-term</th>
<th>Continuous</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Urine excretion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>35.7 ± 3.1 (7)</td>
<td>38.4 ± 5.2 (5)</td>
</tr>
<tr>
<td>Bile duct ligated</td>
<td>88.0 ± 5.8 (3)</td>
<td>65.7 ± 2.2 (5)</td>
</tr>
<tr>
<td>Bile drained</td>
<td>37.0 ± 5.7 (5)</td>
<td>40.0 ± 3.4 (5)</td>
</tr>
<tr>
<td><strong>Biliary excretion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bile drained</td>
<td>47.8 ± 4.0 (5)</td>
<td>55.6 ± 2.0 (5)</td>
</tr>
<tr>
<td>Anephric</td>
<td>82.1 ± 6.4 (4)</td>
<td>67.8 ± 3.8 (5)</td>
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</table>

* Mean ± S.E.

* Numbers in parentheses, number of animals.

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**Chart 3.** Plasma methotrexate level as a percentage of the initial concentration over time, for 4 anephric animals (●), 4 bladder-excised animals (△), 4 anephric-bile-drained animals (○), and 4 bladder-excised-bile drained animals (▲). Bars, S.E.
increased plasma methotrexate levels which occur when this pathway is blocked by bile duct ligation.

The role of absorption and recirculation of biliary methotrexate, the enterohepatic cycle, is less clear. Chung et al. (9) have demonstrated saturable absorption kinetics with a relatively low \( K_m = 1.49 \times 10^{-5} \) m. This is consistent with previous reports of inefficient gut absorption, particularly at high methotrexate levels similar to those attained in this study (5, 13, 16, 18). In our study, urinary excretion under both infusion conditions was the same in bile-drained animals and in normal animals, suggesting that during the 2-hr period of the experiment, intestinal reabsorption of methotrexate did not contribute significantly to renal excretion.

Further evidence that salvage of biliary methotrexate by the enterohepatic cycle does not change plasma kinetics in animals with normal renal function is seen in the plasma clearance curves of methotrexate following bolus injection (Chart 2) and continuous infusion. Clearance of methotrexate after bolus injection is extremely rapid, and as described previously, could be characterized as a 3-component curve (14, 15, 21). The first 2 components of the curve have been associated with the clearance of the drug into the total-body water space and the binding to dihydrofolate reductase (6, 14, 15, 17). It has been postulated that the third component of the curve reflects recycling of the drug via the enterohepatic cycle (14, 15, 17). However, our measurements of plasma clearance in otherwise normal animals undergoing bile drainage provides evidence against this theory (Chart 2). Elimination of enterohepatic recirculation did not alter the plasma clearance curve. Similarly, there was no change in the continuously infused animals, whether bile drained or allowed to reabsorb biliary methotrexate. Since some biliary methotrexate is clearly destined for reabsorption (5, 9, 13, 16, 18), this suggests an interaction with other clearance mechanisms, the most probable being renal.

Such a compensatory interrelationship of renal and biliary elimination routes was demonstrated when one or the other was interrupted. Ligation of the bile duct resulted in increased renal excretion of methotrexate, while nephrectomy increased biliary excretion (Table 1). This was true in both short-term and continuous-infusion animals. This interrelationship was further demonstrated by studies of the effect of bile drainage on plasma methotrexate levels (Experiment 4). In animals with normal renal function, prevention of reabsorption of biliary methotrexate did not affect plasma levels. In contrast, with severely impaired renal function interruption of the enterohepatic cycle dramatically reduced plasma methotrexate levels, the difference being the ability of renal function to compensate for changes in the reabsorption of biliary methotrexate. Since methotrexate toxicity in patients receiving high-dose methotrexate infusions correlates with plasma methotrexate level, the effectiveness of bile drainage in reducing plasma levels in the face of renal failure is potentially of therapeutic importance.

In summary, large amounts of methotrexate are cleared from plasma and excreted into the bile. At plasma methotrexate levels of \( 10^{-6} \) m, biliary clearance of the drug equals or surpasses urinary clearance. In spite of this, prevention of reabsorption of biliary methotrexate (i.e., interruption of the enterohepatic cycle) had little effect on plasma methotrexate levels in animals with normal renal function. This paradoxical result was obtained because of the normal slow and inefficient intestinal absorption of biliary methotrexate at these high concentrations (5, 9, 13, 16, 18) coupled with renal compensation. In rats with severely impaired renal function, biliary diversion is effective in rapidly lowering the plasma methotrexate level. This has potential implications for the clinical management of methotrexate therapy. In patients developing acute renal failure, interruption of the methotrexate enterohepatic cycle by biliary diversion or by enzymatic destruction of the drug in the intestine could help augment clearance from circulation.

REFERENCES

3 Unpublished data.
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