Formation and Elimination of 7-Hydroxymethotrexate in the Rat in Vivo after Methotrexate Administration

Roy M. Brennes, Lars Slørdal, Erik Wist, and Jarle Aarbakke
Department of Pharmacology, Institute of Medical Biology [R. M. B., L. S., J. A.], and Department of Oncology, Institute of Clinical Medicine [E. W.], University of Tromsø, N-9001 Tromsø, Norway

ABSTRACT

Bile, urine, and serum concentrations of methotrexate (MTX) and 7-hydroxy-methotrexate (7-OH-MTX) were monitored in rats in vivo following a short-time infusion of 10 mg/kg [3H]MTX. The experiments were performed in one group of anesthetized, bile-drained rats and in two control groups, one anesthetized and one unanesthetized, that were not bile-drained. Peak biliary levels of MTX (3.8 × 10^3 m) and 7-OH-MTX (1.8 × 10^4 m) appeared within 15 min after cessation of infusions. For two log ranges of serum MTX concentrations, biliary levels remained 180-fold higher. High bile 7-OH-MTX levels appeared few min after start of MTX administration, and were 720 times higher than the peak serum concentrations, indicating that the liver is a major site of 7-OH-MTX formation in the rat. 7-OH-MTX concentrations in bile declined monophasically with a half-life of 29.4 min, while MTX showed a biphasic elimination with initial and second phase half-lives of 23.1 and 86.4 min, respectively. Bile was the major excretory route for MTX and 7-OH-MTX, with 50% of the dose recovered as the parent compound and 3.6% as the metabolite. There was no difference in urinary recovery of MTX in bile-drained and control animals, indicative of insignificant enterohepatic circulation of MTX. This was further corroborated by the finding of just 2.1% urinary recovery of MTX in rats who received previously collected MTX-containing bile through a duodenal catheter. Serum concentration curves were analyzed according to a three-compartment open model with an initial elimination half-life of 1.7-3.3 min, a second phase half-life of 15.4-21.0 min, and a terminal phase half-life of 119-240 min. Our finding of 7-OH-MTX formation and high biliary levels of the metabolite in the rat, can be used as basis for studies of interactions with in vivo MTX conversion to the 7-hydroxy metabolite.

INTRODUCTION

The antifolate agent MTX is widely used in the treatment of malignancies in humans, and HD-MTX infusions are currently employed in several therapy regimens (1). In humans, MTX is in part metabolized to intracellularly confined polyglutamates (2), and to considerable amounts of 7-OH-MTX (3-7) and small amounts of DAMPA (8). In addition to renal excretion, MTX is subjected to biliary elimination (4, 9). 7-OH-MTX has been demonstrated to interact with the pharmacokinetics, and possibly the pharmacodynamics, of MTX by affecting the latter with respect to intracellular entry, polyglutamation, and efflux (3, 10-12). The metabolite is in addition highly insoluble in aqueous solutions, and has been implicated as a possible mediator of potentially fatal renal failure during HD-MTX therapy (13-15). To further evaluate its significance, we have initiated a series of studies on in vivo MTX pharmacokinetics in a rodent model.

The rabbit has, presumably due to a very high degree of metabolic conversion of MTX to 7-OH-MTX, been employed in several studies of MTX and 7-OH-MTX pharmacokinetics (16-20). Based on the gross diversity between MTX conversion rates in human and rabbit hepatic tissues in vitro (21), the applicability of a rabbit model may seem questionable. Rat hepatocytes and extracts of rat liver have, however, been demonstrated to cause biotransformation of MTX to 7-OH-MTX at a rate which is more in agreement with comparable data from experiments employing human tissue (21-23). Apart from a single previous report of detectable serum and urine concentrations of 7-OH-MTX after administration of 1 mg/kg MTX in rats (24), there are hitherto no comprehensive studies on in vivo 7-hydroxylation in this species.

Biliary excretion has been reported to be of considerable importance for the elimination of MTX in the rat (25-29). In humans, measurements of MTX in bile have yielded inconclusive data, with reports of biliary MTX recovery ranging from 0.4 to 20% of the dose administered (9, 30). Furthermore, the significance of a potential enterohepatic circulation of MTX has not been established.

In the present study, we have therefore investigated the in vivo conversion of MTX to 7-OH-MTX, the enterohepatic circulation of MTX, and the pharmacokinetics of the metabolite and the parent compound after a short-time infusion of 10 mg/kg [3H]MTX in the rat.

MATERIALS AND METHODS

Drugs and Chemicals. L-Glutamyl-3,4-[3H]MTX (specific activity, 36.0 Ci/mmol; purity, >99% by HPLC) was purchased from New England Nuclear, Boston, MA. Unformulated MTX (purity, >99% by HPLC) was a gift from Nycomed A/S, Oslo, Norway. 7-OH-MTX was a gift from Dr. W. E. Evans, St. Jude Children's Research Hospital (Memphis, TN). Hypnorm vet. (fentanyl, 0.2 mg/ml; fluanisone, 10 mg/ml) was from Janssen Pharmaceutical, Beersel, Belgium. Insta-gel II scintillation liquid was from Packard Instruments Company, Groningen, The Netherlands. Methanol and tetrahydrofuran (both HPLC grade) were from Rathburn Chemicals, Walkenburn, UK. All other reagents were of analytical grade. All samples containing MTX and 7-OH-MTX were stored protected from light at -20°C for a maximum of 2 weeks.

Animals and Operations. Male Wistar rats weighing 220-300 g (obtained from Mjøllegaard Hansens Avlslaboratorier A/S, Ejby, Denmark) were used for the experiments. The rats were randomly allocated to four groups (A-D), each of which consisted of four to six animals. Animals in group A (n = 6) were anesthetized and bile-drained during the experiments. Group B (n = 6) served as anesthetized controls during experiments. Three animals of group D (n = 6) were bile-drained while their three litter mates received indwelling duodenal catheters. All animals in group D were anesthetized during experiments. None of the animals in group B and C were bile-drained. Under fentanyl anesthesia (0.3 mg/kg i.p.), all the rats had the external jugular vein cannulated unilaterally with a 1.26-mm polyethylene catheter. During this initial procedure, animals to be bile-drained underwent laparotomy, the bile duct was isolated and cannulated with a 0.63-mm polyethylene catheter prior to immediate transfer to restraining cages for experiments. Group B animals were transferred to cages for experiments immediately after cannulation of the vein. The
venous catheters of group C animals were secured as described elsewhere (31), and these animals recovered overnight before placement in restraining cages for experiments.

Group D animals underwent venous cannulations as described. During initial anesthesia the donor animals had their bile duct cannulated, while cannulation of the duodenum was performed in the recipient animals by laparotomy and a small incision in the duodenum approximately 10 mm from the pylorus. These animals were immediately transferred to restraining cages for experiments.

Experiments. Group A, B, C, and the donor animals of group D each received an i.v. short term infusion of [3H]MTX (10 mg/kg, 0.114 mCi/kg) in isotonic saline with 0.1 M NaOH, administered through the central venous catheter during 10 min. The venous catheters were flushed with heparinized (10 IU/ml) saline immediately after administration of drug, and after each sampling. Venous samples of 200 µl were drawn from the catheters at scheduled intervals starting right after cessation of drug infusion and until 10 h (Fig. 4). Bile samples were obtained during the period of MTX infusion (10 min), during 15-min intervals for the initial 60 min, during 30-min intervals for the next 60 min, during 60-min intervals between 2 and 8 h, and one 2-h sample between 8 and 10 h (Fig. 1). To investigate the significance of enterohepatic circulation of MTX, 3 recipient rats received intraduodenally precollected bile from 3 MTX treated (10 mg/kg) bile-drained donor animals. Bile was collected from the donors at intervals as described above. After removing an aliquot of 50 µl for determination of MTX concentration, the bile was immediately infused in the recipients’ duodenum. Venous samples of 200 µl were drawn at scheduled intervals until 8.5 h (Fig. 3).

With the exception of group C, all animals received maintenance fentanyl anesthesia, approximately 0.08 mg/kg/h, administered as single i.m. injections every hour. All anesthetized animals were hydrated with 0.06 M NaHCO3 in isotonic saline, approximately 10 ml/kg/h and 6 ml/kg/h to bile-drained and control animals, respectively. Group C animals were permitted laboratory chow and water ad libitum.

During experiments, voided urine was collected from all rats. Upon sacrificing the animals, the urine bladder was aspirated to assure complete collection. pH was measured in voided urine samples. Venous blood gas and hematocrit samples were drawn from the venous catheters at 6 and 10 h.

Analytical Methods. Analysis of MTX and 7-OH-MTX concentrations in serum were performed by reversed-phase HPLC (32), with the following slight modifications: The mobile phase was 17.6% (v/v) methanol and 0.4% (v/v) tetrahydrofuran in tris-sodiumhydrogen-phosphate (both 0.1 M, pH 6.7), and the column a Supelcosil LC-18 150 × 4.6 mm, 3 µm (Supelco Inc., Bellefonte, PA). The eluent was monitored at 370 nm. The assay detects both MTX and its major extracellular metabolites 7-OH-MTX and DAMPA, with no interference from polyglutamates 1–3 of MTX.

After thawing, 100-µl aliquots of serum, urine, and bile were mixed with 25 µl of perchloric acid (2 m) (50 µl bile aliquots of group D animals were mixed with 12.5 µl perchloric acid), and the samples were centrifuged at 10,000 × g for 10 min. Supernatants of serum samples were siphoned off and injected on the chromatograph. Supernatants of urine and bile were diluted 1:10 (v/v) in distilled water before injection. Urine and bile samples were in addition analyzed by fraction sampling and determination of radioactivity. The eluent was sampled as 20-s fractions in 10-ml counting vials before addition of 10-ml scintillation liquid. Radioactivity was determined by a Packard MINIAXIS/3 Tribloc liquid scintillation spectrometer, Series 4000 (Packard Instrument Company, La Grange, IL), using an automatic program for 3H-determination which also corrects for quenching. Assuming that radioactivity detection measures 100% of MTX in the fraction samples, the recoveries by HPLC analysis with UV detection in bile and urine were 90 ± 13 (n = 82) and 98 ± 4% (n = 22) (mean ± SD), respectively, over a concentration range from 0.45 to 2200 µM.

Calculations. Concentrations of 7-OH-MTX and MTX versus time were plotted on semilogarithmic graphs. The serum concentrations were analyzed according to a three-compartment model. Pharmacokinetic parameters were obtained from successive curve peeling by means of linear regression analysis in a semilogarithmic data set, and refer to the triexponential equation:

\[ C = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t} \]  

(A)

**RESULTS**

7-OH-MTX and MTX were recovered in bile within few minutes after initiation of the MTX infusions, with peak biliary concentrations within 15 min after termination of infusions (Fig. 1). Biliary flow was fairly constant at 0.96 ± 0.19 ml/h (n = 54) during experiments (Fig. 1). The mean peak concentrations in bile of MTX and 7-OH-MTX were 3.8 × 10⁻³ M and 1.8 × 10⁻⁴ M, respectively. During the first 15 min after drug administration there was a 720-fold concentration of 7-OH-MTX in bile over serum. There was a linear correlation between the concentration of MTX in bile and the simultaneous MTX serum level described by:

\[ [\text{MTX}_{\text{BILE}}] = 178 \times [\text{MTX}_{\text{SERUM}}] \]  

(C)

(n = 42, r = 0.95) (Fig. 2), i.e., a 178-fold concentration of MTX in bile over serum within the first 3 h and 2 log ranges of the experiments. In a semilogarithmic plot, following the peak at 15 min, 7-OH-MTX and MTX concentrations declined rapidly with a monophasic curve for the 7-hydroxy metabolite and a biphasic curve for the parent compound. The 7-OH-MTX half-life was 29.4 min (r = 0.99), and the MTX initial and second phase half-lives were 23.1 (r = 0.99) and 86.4 (r = 0.95) min. Of the administered MTX dose, 50.0 ± 14.8% was excreted in bile as MTX and 3.57 ± 0.75% as 7-OH-MTX (Table 1). Of the total MTX excreted in bile, 97.4% and 99.8% was excreted within 2 and 6 h after the infusions, respectively.

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**Fig. 1.** Biliary concentration of methotrexate (O) and 7-hydroxy-methotrexate (●) following short-time infusions of 10 mg/kg [3H]methotrexate to six anesthetized rats. Insert, biliary flow-rate during the experiments. Data are given as mean ± SD.
Fig. 2. Relationship between serum and bile methotrexate concentrations in six rats during the first 3 h following short-time 10 mg/kg [3H]methotrexate infusions. [MTX<sub>B</sub>] = 178 × [MTX<sub>S</sub>], n = 42, r = 0.95.

Table 2 Urinary methotrexate recovery as methotrexate and 7-hydroxy-methotrexate in bile-drained and control rats

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Methotrexate (%)</th>
<th>7-Hydroxymethotrexate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>6</td>
<td>16.0 ± 11.8</td>
<td>25.9 ± 2.8</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>11.2 ± 15.4</td>
<td>30.0 ± 8.1</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>30.8 ± 11.6</td>
<td>31.1 ± 11.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.05 between group A and B.
<sup>b</sup> P < 0.05 between group B and C.

There was no statistically significant difference between group A, B, and C with regard to the urinary recovery of administered MTX (Table 2). The volumes of urine voided were practically identical in the groups of anesthetized animals, but significantly larger in the unanesthetized. Within 6 h of drug infusion, 63.1% of the urinary recovered MTX (10 h) was found in the urine in group A, 32.5% in group B, and 99.2% in group C, with statistically significant differences between group B and C only. Significantly more 7-OH-MTX was detected in urine within 6 h in group A as compared to group B, while significantly less 7-OH-MTX was measured in urine from group B upon comparison with group C.

In the three animals administered MTX- and 7-OH-MTX-containing bile by the route of a duodenal catheter, monitoring of serum showed detectable MTX concentrations 15 min after the initial bile infusion (Fig. 3). Peak serum MTX concentrations (0.105 ± 0.018 µM) were detected at 105 min following the first administration of MTX-containing bile. 7-OH-MTX was not detected in the serum samples examined. Urinary recoveries of the MTX and 7-OH-MTX amounts in the intraduodenally administered bile were 2.11 ± 0.82 and 1.45 ± 1.01%, respectively, within 8.5 h. Because of the small fraction of MTX absorbed and the disproportionately low urinary recovery of 7-OH-MTX as compared to the parent compound, it is assumed that negligible formation of 7-OH-MTX took place in the recipients. Consequently, we have not accounted for possible metabolism of the parent compound in these calculations.

The serum concentrations of MTX declined rapidly over the initial 5–10 min, followed by a slower second phase for approximately 3 h prior to a much slower third phase (Fig. 4). Triexponential curves were fitted to the data, and pharmacokinetic parameters were calculated according to a three-compartment model with elimination from the central compartment. The pharmacokinetic variables are given in Table 3. MTX concentrations declined more rapidly in bile-drained animals of group A, who demonstrated shorter half-lives compared to their controls in group B. Animals in group C had a more rapid decline of serum MTX concentrations, with shorter second and third phase half-lives than group B. The apparent volume of distribution in the postdistributional phase, \( V_f \), was significantly larger in group B than in group A, reflecting a significantly longer terminal half-life in group B, while there was no difference between the two groups with regard to \( V_c \) (the apparent central volume of distribution). In group B, \( V_f \) was significantly larger, while the initial half-life and \( V_c \) were significantly smaller than in group C. Group C animals tended to a higher total clearance (\( Cl_T \)) than group A and B, but the difference was not significant. There was no difference in \( Cl_T \) between groups A and B.

Venous pH, measured by blood gas analysis at 6 h after drug infusion, was in the range of 7.41–7.46 in all animals. Hematocrit values in group C at 6 h (0.33 ± 0.02) and 10 h (0.29 ± 0.01) were significantly higher than hematocrits in the anesthetized groups A and B. pH in urine of all animals increased slowly, presumably as a result of NaHCO<sub>3</sub> administration, and reached pH 7 within 4 h of drug infusion in group C and within 7 to 8 h in groups A and B.

DISCUSSION

Most animal studies of MTX conversion to 7-OH-MTX have been carried out in rabbits (16–20). Previous investigations in an isolated perfused rat liver model showed that MTX passed through the liver unmetabolized (28), and later Chen & Chiou (17) have stated that hydroxylation of MTX was absent in rats.
Here, we demonstrate that a rat model can be applied for in vivo studies of 7-OH-MTX formation and excretion.

In our experiments, 7-OH-MTX and MTX appeared in bile at high concentrations during the 10-min MTX infusions, and peak concentrations were measured 15 min after cessation of infusions (Fig. 1). The in vivo pattern of MTX excretion is therefore considerably more rapid than the 40-min lag between infusion and secretion described in the isolated perfused rat liver (28). Since bile flow was constant (Fig. 1), biliary concentrations reflect rates of biliary elimination of MTX and biliary formation and elimination of 7-OH-MTX. Both compounds were eliminated rapidly from bile with initial half-lives of less than 30 min, and of the total MTX recovered in bile, 87.2% was excreted within the first hour.

Consistent with previous studies in rats (28) and humans (33), we found MTX bile concentrations surpassing serum concentrations 178-fold. Steinberg et al. (29) described a considerably lower bile:serum ratio (27:1) in rats receiving 250 mg/kg MTX, but demonstrated a linear correlation between bile and serum concentrations in agreement with our results (Fig. 2).

The bile:serum ratio of 7-OH-MTX at peak serum concentrations was four times higher than the corresponding MTX ratio. Although the enzyme responsible for MTX 7-hydroxylation in the rat remains unidentified (21, 22, 34), previously published data suggest that hepatic parenchymal cells may play an important role in the biotransformation (23). The high 7-OH-MTX bile:serum ratio, as well as the presence of significant concentrations of the metabolite in bile only few minutes after initiation of the MTX infusion, support the notion that the liver is a major site of biotransformation of MTX to 7-OH-MTX in the rat.

In our study, the 7-OH-MTX recovery as 3.5% of the MTX dose is markedly higher than the 1.7% bile recovery during 6 h sampling in rabbits, as reported by Sasaki et al. (16). In that study, 32% of the administered dose was recovered from tissues, gastrointestinal tract, feces, urine, and bile as 7-OH-MTX following a 6-h infusion of 50 mg/kg MTX. In the rabbit, several tissues convert MTX to the 7-hydroxy metabolite (16), and rabbit liver extracts have a conversion rate considerably higher than rat and human hepatic extracts (21). It is further noteworthy that the recovery of the 7-hydroxylated metabolite of MTX in the rat is in much closer agreement with the biotransformation in humans, reported to be in the 0.4–20% range (4, 35), when compared to the 32% 7-OH-MTX conversion in the rabbit.

Consistent with other investigators, we have demonstrated a triphasic plasma disappearance curve of MTX (29, 36, 37). The rat shows a dose-dependent MTX elimination with a triphasic elimination curve of MTX in serum after MTX doses exceeding 3.1 mg/kg (37). Our data show that the initial elimination phase is due to distribution of MTX into tissues, and to some extent excretion into bile and urine, while the second phase primarily represents drug elimination via biliary and renal excretion. The third phase is consistent with postdistributional equilibrium representing intracellular confinement of MTX as polyglutamates and strong binding to target enzymes. However, the third phase has been postulated to represent enterohepatic recycling of the drug (33, 38). Despite similar urinary excretion in normal and bile-drained animals, the observed small gastrointestinal resorption of MTX may be of importance in the terminal phase of elimination when serum concentrations are low, reflecting small amounts of drug in the body. The finding of a shorter terminal half-life and a smaller Vt of bile-drained animals compared to their controls is consistent with this interpretation.

Contrary to the findings of Steinberg et al. (29) in unanesthetized rats, we observed significantly shorter half-lives in bile-drained animals compared to their controls. We assume that physiological changes due to anesthesia play a role in this diversity. In unanesthetized rats we found a significantly faster renal excretion, which in turn affected pharmacokinetic parameters. This may be explained by accelerated alkalinization and possibly by a significantly higher urine flow in the unanesthetized animals (39). Our data on Vc and Clr are in concordance with others (24, 36). Of the administered MTX, very small
amounts were recovered in urine as 7-OH-MTX.

Comparable urinary recovery of MTX in normal and bile-drained animals is consistent with the findings of Steinberg et al. (29) and indicates a low-grade intestinal resorption of biliary MTX. Further, it is consistent with our finding of a small (2.1%) urinary recovery of MTX after intraduodenal administration of MTX-containing bile to litter mates. There are previous reports of inefficient gut absorption of MTX (26, 28, 40, 41), and saturable absorption kinetics of MTX in the intestinal tract has been demonstrated at a relatively low Km value of 1.49 × 10^-5 M (42). Previous studies in humans (33, 38) and rats (37), have suggested the MTX, which is not recovered in urine, to be excreted into bile and feces or stored intracellularly as polyglutamates. In bile-drained animals, we could account for 86% of the administered dose during a 10-h sampling period. As outlined above, the small gastrointestinal absorption indicates a significant amount of MTX and 7-OH-MTX to appear in the feces in rats with an intact bile duct. This is consistent with previous findings in the rat with 29–41% of the administered dose has been recovered in the stool after 25 mg/kg MTX i.p. (25).

In summary, MTX is rapidly converted to 7-OH-MTX in rats administered 10 mg/kg of [3H]MTX. High concentrations of MTX and 7-OH-MTX appear in bile within few minutes, suggesting the liver to be a major site of MTX conversion. Similar urinary MTX recovery between groups with and without biliary drainage and a slight gastrointestinal resorption of MTX, indicate that enterohepatic cycling of MTX contributes little to renal excretion at the dose used. The present data represent a basis for studies of interactions with in vivo 7-OH-MTX formation and biliary secretion in rats given MTX.

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