Dose-dependent Pharmacokinetics of Methotrexate and 7-Hydroxymethotrexate in the Rat in Vivo

Roy M. Bremnes, 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

The pharmacokinetics of methotrexate (MTX) and 7-hydroxymethotrexate (7-OH-MTX) in bile, urine, and serum was studied in rats in vivo after short-time infusions of 10, 50, 250, and 1000 mg/kg MTX. All animals were anesthetized and drained of bile during experiments. The biliary secretion rate of MTX approached saturation when serum MTX levels surpassed 700–800 μM, causing a significant reduction in biliary recovery as the parent compound (49 to 32%) at MTX doses exceeding 50 mg/kg. The hepatic metabolism of MTX to the 7-hydroxy metabolite was not saturated at the doses used. Serum MTX pharmacokinetics demonstrated dose dependency, inasmuch as doses exceeding 10 mg/kg were accompanied by a reduced total body clearance (ClT) and biliary clearance (ClB). A significant finding in relation to acute hepatotoxicity reported after high-dose MTX in humans was the occurrence of cholestasis 30–90 min after drug infusion and the observation of macroscopic precipitations in the bile duct in five of six animals treated with 1000 mg/kg MTX. In these five animals, cessation of bile secretion occurred at similar bile 7-OH-MTX levels (9600 ± 1100 SD μM), while the single rat that secreted bile throughout the experiment had a 5-fold lower peak 7-OH-MTX concentration in bile. Analysis of biliary precipitates formed in vivo and in vitro found 7-OH-MTX to constitute 97% and MTX 3% of the drug content of the precipitated material.

INTRODUCTION

The antifolate agent MTX4 is widely used in cancer chemotherapy, and HD-MTX (1–34 g/m²) infusions are currently used in several therapy regimens (1). In humans, MTX is in part metabolized to 7-OH-MTX, which is measured in high concentrations in the blood after HD-MTX infusions (2–12). 7-OH-MTX has been demonstrated to be cytotoxic and to affect cellular entry, polyglutametation, and efflux of the parent compound in vitro (3, 13–17). The limited aqueous solubility of the metabolite, when compared to the parent drug, has rendered it a possible mediator of renal toxicity during HD-MTX therapy (18, 19).

There are numerous reports on acute hepatotoxicity after HD-MTX therapy (20–29) and chronic hepatotoxicity due to long-term low-dose MTX treatment for acute leukemia and autoimmune diseases (30–44). It has been postulated that the hepatotoxicity of MTX is caused by a toxic metabolite (45, 46), but the underlying mechanisms that cause liver injury to occur after MTX therapy remain unknown.

Compared to the frequently applied rabbit model for evaluation of MTX metabolism and 7-OH-MTX pharmacokinetics (47–52), data obtained in the rat are in better agreement with findings in humans (53–55). Furthermore, the rat seems to be the only animal model of human chronic MTX hepatotoxicity (56).

Previously, we studied the in vivo 7-hydroxylation in the rat after 10 mg/kg [³H]MTX (57). Herein we report the dose dependency of MTX and 7-OH-MTX pharmacokinetics after short-time infusions of MTX in doses ranging from 10 to 1000 mg/kg, which cover the range of HD-MTX doses used in the clinic.

MATERIALS AND METHODS

Drugs and Chemicals. L-Glutamyl-3,4-[³H]MTX (specific activity, 37.1 Ci/mmol; purity, 99.2% by HPLC) was purchased from New England Nuclear, Boston, MA. Unformulated and formulated MTXs (purity, 99% by HPLC) were gifts from Nycomed A/S, Oslo, Norway. 7-OH-MTX was a gift from Dr. F. M. Sirotnak, Memorial Sloan-Kettering Cancer Center, New York, NY. Hypnorm vet. (fentanyl, 0.2 mg/ml; fluanisone, 10 mg/ml) was from Janssen Pharmaceutical, Beerse, Belgium. Heparin was obtained from Nycomed A/S, and NaHCO₃ was from Naflab Hospital A/S, Oslo, Norway. Insta-Gel II scintillation liquid was from Packard Instruments Co., Groningen, The Netherlands. Methanol and tetrahydrofuran (both HPLC grade) were gifts from Rathburn Chemicals, Walkenburn, United Kingdom. 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 4 weeks.

Animals and Operations. Male Wistar rats weighing 250–370 g (obtained from Charles River, WIGA GmbH, Sulzseid, West Germany) were used for the experiments. The rats were randomly allocated to four groups (A, B, C, and D), each of which consisted of 6 animals. All animals were anesthetized and drained of bile during the experiments. Under fentanyl (0.3 mg/kg i.p.) anesthesia, all animals had their bile duct and right external jugular vein cannulated as described previously (57).

Experiments. Methotrexate solutions were prepared by dissolving the drug in isotonic saline with 0.1 M NaOH to concentrations of 1, 5, 25, and 100 mg/ml MTX, thus allowing administration of equal volumes to all rats. Tritiated MTX was added to an activity of 9.2 μCi/ml at each concentration applied. [³H]MTX doses of 10, 50, 250, and 1000 mg/kg were administered to group A, B, C, and D animals, respectively. The drug was administered as a continuous infusion through a central venous catheter for 10 min. The venous catheters were flushed with heparinized (10 IU/ml) saline immediately after administration of drug and after each subsequent blood sampling. Venous samples of 200 μl were drawn from the catheters as follows. Prior to and immediately after drug administration and subsequently 2, 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 min after cessation of MTX infusion. Bile samples were obtained prior to and during the period of MTX administration (10 min), during 15-min intervals for the initial 60 min, during 30-min intervals for the next 60 min, and during 60-min intervals between 2 and 6 h. Voided urine was collected from all rats, and upon sacrificing the animals, the urine bladder was aspirated to assure complete collection. pH was measured in voided urine and bile samples. All animals received maintenance fentanyl anesthesia, approximately 0.08 mg/kg/h, administered as single i.m. injections every hour and were hydrated with 8 ml/kg/h of 0.06 M NaHCO₃ in isotonic saline. Venous blood gas and hematocrit samples were drawn from the venous catheters immediately prior to MTX treatment and at the end of the experiments.
Analytical Methods. Analysis of MTX and 7-OH-MTX concentrations in serum were performed by reverse phase HPLC as reported previously (57). The assay detects both MTX and its major extracellular metabolites 7-OH-MTX and 2,4-diamino-7-N\textsubscript{2}-methylpterioic acid, with no interference from polyglutamates 1–3 of MTX. Urine and bile samples were analyzed by fraction sampling and determination of radioactivity (57). Assuming that radioactivity detection measures 100% of MTX in the fractionated samples, the recoveries by HPLC analysis of bile and urine were 94 ± 7 (SD) (n = 195) and 90 ± 8 (n = 49), respectively, over a concentration range from 1.8 μM to 27 mM.

Calculations. Concentrations of 7-OH-MTX and MTX versus time were plotted on semilogarithmic graphs. The serum concentrations were analyzed according to a two-compartment open model. Pharmacokinetic parameters were obtained by means of linear regression analysis in a semilogarithmic data set and refer to the biexponential equation

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

Total clearance, \( Cl_T \), was calculated by the equation

\[ Cl_T = \frac{Dose}{(AUC_0 + A/\alpha + B/\beta)} \]

where \( AUC_0 \) is the area under the curve during drug infusion (10 min), calculated by a triangular area. \( A \) and \( B \) are the zero-time intercepts of the extrapolated lines of the \( \alpha \) and \( \beta \) phases, respectively.

Biliary clearance was calculated by the equation

\[ Cl_B = \frac{Bile \ flow \cdot C_B}{C_S} \]

where \( C_B \) and \( C_S \) are corresponding concentrations in bile and serum, respectively. \( C_B \) values are obtained from bile sampled for 15, 30, or 60 min, whereas \( C_S \) values are calculated means of serum concentrations at start and cessation of each bile sample interval. The central volume of distribution, \( V_C \), was obtained by dividing the dose by (\( A + B \)), and the apparent volume of distribution in the postdistributional phase, \( V_P \), was calculated by dividing total clearance by \( \beta \).

All results are expressed as mean ± SD. Statistical analyses were performed by one-way analysis of variance and estimation of least significant distance (Statgraphics; STSC, Rockville, MD). Statistical significance was defined as \( P < 0.05 \).

RESULTS

The results obtained after administration of 10 mg/kg MTX essentially reproduce our previous findings at this dose level (Tables 1–4; Figs. 1 and 5) (57).

The bile flow during the first hour of the experiments was significantly larger in group C than in groups A and B (Table 1). The biliary pH, however, remained constant in all animals throughout the experiments. Bile MTX concentrations declined monophasically in a semilogarithmic plot (Fig. 1), with a significantly longer half-life in group C, 55.6 min (mean), than in groups A and B. Peak bile MTX concentrations increased as a function of the administered MTX doses, but in a nonlinear manner. Whereas a dose escalation from 10 to 50 mg/kg resulted in a mean 180% increase in peak bile MTX levels, dose increments to 250 and 1000 mg/kg were accompanied by stepwise increases of 22 and 18%, respectively. As shown in Fig. 2, the biliary secretion rate approached saturation when serum levels surpassed 700 μM MTX in animals given 250 mg/kg MTX. Furthermore, a significant reduction in biliary clearance of MTX at a dose escalation from 50 to 250 mg/kg led to a correspondingly smaller biliary recovery of unaltered drug (Tables 2 and 4).

In group C animals, 7-OH-MTX concentrations in bile declined monophasically with a 49.7-min (mean) half-life, which was significantly longer than in groups A and B (Fig. 1). As disposition kinetics seemed to approach an upper threshold concerning the ability to secrete and concentrate MTX in the bile, the biliary 7-OH-MTX concentrations increased 4- and 3-fold with dose increments from 10 to 50 and from 50 to 250 mg/kg, respectively (Fig. 1). However, in group D (1000 mg/kg) cessation of bile secretion occurred within 30–90 min after

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**Fig. 1.** Biliary concentrations of methotrexate (○) and 7-hydroxymethotrexate (●) versus time following short-time infusions of 10 (A), 50 (B), and 250 (C) mg/kg [3H]methotrexate in rats. All animals were anesthetized. Data are given as mean ± SD, \( n = 6 \).
MTX and 7-OH-MTX were removed during the total washing procedure. More MTX than 7-OH-MTX was removed during the first washing with NaCl, while more 7-OH-MTX was removed during the second. Of the total MTX and 7-OH-MTX content in the pellets, 97.2 ± 0.9% (N = 6) was 7-OH-MTX (Fig. 4). The in vivo precipitation of 7-OH-MTX in bile was reproduced in vitro when the 7-hydroxylated metabolite and the parent compound, both at a concentration of 9000 μM, were added to freshly collected rat bile. Of the drug content in the resulting precipitate, 97.1% was constituted by 7-OH-MTX.

The urinary recovery of unaltered drug and nonbiliary (urinary and metabolic) clearance remained unchanged at dose increments (Tables 3 and 4). Whereas the MTX recovery as the 7-hydroxy metabolite in urine (Table 3) and the volumes of urine voided (Table 1) were significantly larger in animals of group D compared to those in groups A–C. In all animals, urine pH increased slowly as a result of alkalinization with NaHCO₃. Only group D animals reached pH 7 within the 6-h experimentation period.

In serum, the MTX concentrations declined rapidly in each group of animals over the initial 10 min, followed by a slower second phase (Fig. 5). The pharmacokinetic variables are given in Table 4. Doses exceeding 10 and 50 mg/kg MTX led to a dose-dependent decline in total body clearance (ClT) and biliary clearance (Clb). Animals in group D demonstrated the longest t₁/₂ and largest Vc and Vf. The peak serum 7-OH-MTX concentrations in group D animals (300 ± 210 μM), which appeared after onset of cholestasis in 5 of the rats, were considerably higher than in the other groups (means, 0.34–16.8 μM). After reaching peak levels, serum 7-OH-MTX declined monophasi-

### Table 2 Biliary methotrexate recovery as methotrexate and 7-hydroxy-methotrexate in bile-drained rats

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Dose (mg/kg)</th>
<th>Methotrexate (%)</th>
<th>7-Hydroxy-methotrexate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6</td>
<td>10</td>
<td>50.3 ± 5.6°</td>
<td>52.5 ± 5.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>47.7 ± 0.9°</td>
<td>47.8 ± 8.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>25.9 ± 6.3</td>
<td>32.4 ± 9.8</td>
</tr>
</tbody>
</table>

° P < 0.05 between 10 and 50 mg/kg.

Table 3 Urinary methotrexate recovery as methotrexate and 7-hydroxy-methotrexate in bile-drained rats

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Dose (mg/kg)</th>
<th>Methotrexate (%)</th>
<th>7-Hydroxy-methotrexate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6</td>
<td>10</td>
<td>27.4 ± 4.6</td>
<td>0.53 ± 0.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>23.1 ± 6.0</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>30.7 ± 14.2</td>
<td>0.06 ± 0.06</td>
</tr>
<tr>
<td>D</td>
<td>5°</td>
<td>1000</td>
<td>30.8 ± 14.2</td>
<td>3.7 ± 2.3°</td>
</tr>
</tbody>
</table>

° One animal that died 30 min after cessation of bile secretion has been excluded.

* P < 0.05 between 250 and 1000 mg/kg.
MtX. In rats infused with 250 mg/kg MtX the maximal rate of transport ($T_{max}$) and the transport constant analogous to the Michaelis-Menten-type kinetics at high dose levels of MtX is consistent with a previous investigation by Kates and Tozer (61). The biliary transport system following HD-MTX therapy in humans indicates that 7-OH-MtX is implicated in acute hepatotoxicity underling this mechanism remains unknown. Our results may in fact correspond to a dose of 33 g/m² in an average 8-year-old child. MTX doses up to 33.6 g/m²/24 h have been used in HD-MTX treatment of children with acute lymphoblastic leukemia (9, 20-29). In this patient's previous MTX courses without observed hepatotoxicity, it has further been postulated that hepatotoxicity after doses ranging from 10 to 1000 mg/kg. All animals were anesthetized and drained of bile. Data are given as mean ± SD.

### DISCUSSION

This is the first report concerning precipitation of 7-OH-MtX in the bile of any species. It has previously been established that the 7-hydroxy metabolite is highly insoluble in aqueous solutions and 7-OH-MtX has been found to precipitate in the renal tubules of monkeys (18, 19). Herein, we have demonstrated that the metabolite precipitates at high concentrations in the alkaline rat bile, presumably causing the cholestasis observed in rats treated with 1000 mg/kg MtX. This dose corresponds to a dose of 33 g/m² in an average 8-year-old child. Since doses up to 33.6 g/m²/24 h have been used in HD-MTX treatment of children with acute lymphoblastic leukemia (9, 58-60), these findings may be of clinical relevance. MTX doses of this magnitude are administered as 24-h infusions in humans rather than short-time infusions as in our animal model. Even so, peak serum concentrations of 7-OH-MtX have been detected at 337 µM (9) which exceed our measurements in rats given the equivalent dose of MtX.

There have been several reports on acute hepatotoxicity with elevated serum aspartate aminotransferase and alanine aminotransferase after HD-MTX treatment in humans (20-29). In one patient who developed serious acute hepatotoxicity, Breithaupt and Kuenzlen (27) found inconspicuous plasma MTX kinetics while the 7-OH-MTX kinetics deviated compared to this patient’s previous MTX courses without observed hepatotoxicity. It has further been postulated that hepatotoxicity after MTX is caused by a toxic metabolite of MtX (45, 46), but the underlying mechanism remains unknown. Our results may indicate that 7-OH-MTX is implicated in acute hepatotoxicity following HD-MTX therapy in humans.

Saturation of biliary MtX secretion at serum levels surpassing 700-800 µM MtX is consistent with a previous investigation by Kates and Tozer (61). The biliary transport system displays Michaelis-Menten-type kinetics at high dose levels of MtX. In rats infused with 250 mg/kg MtX the maximal rate of transport ($T_{max}$) and the transport constant analogous to the Michaelis constant ($k_T$) were 20.7 mg/h and 411 µM (means), respectively, somewhat higher than the findings of Kates and Tozer (61), 12 mg/h and 70 µM. Our experiments, however, were not carried out under steady state conditions which are essential for investigations of Michaelis-Menten kinetics, as discussed by Kates and Tozer (61). The saturation of transport explains the disproportional increase of peak MTX concentrations in bile and the significant reduction in biliary clearance of the drug at MTX doses beyond 50 mg/kg, the significantly longer biliary MtX and 7-OH-MtX half-lives following 250 mg/kg MtX, and the successively smaller biliary MTX recoveries with dose increments. However, the hepatic metabolism...
In summary, biliary excretion of MTX approaches saturation at MTX doses exceeding 50 mg/kg, whereas 7-OH-MTX formation and excretion do not. High bile 7-OH-MTX concentrations lead to in vivo cholestasis, presumably due to precipitation in bile where 7-OH-MTX constitutes 97% and MTX 3% of the drug content in vivo and in vitro. The results indicate that 7-OH-MTX may be implicated in acute hepatotoxicity reported after HD-MTX therapy in humans, and they suggest that there may be an upper limit of MTX doses regardless of leukovin rescue.

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