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

Roy M. Bremnes, Lars Slordal, Erik Wist, and Jarle Aarbakke

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 (Clb) 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 (9800 ± 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, polyglutamation, 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 from Rathburn Chemicals, Walkernburn, 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, Sulziseld, 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-NH₃-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, \( V_{\alpha} \), was obtained by dividing the dose by \((A + B)\), and the apparent volume of distribution in the postdistributional phase, \( V_{\beta} \), 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 (Statagraphics; 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 the apparent volume of distribution in the postdistributional phase, \( V_{\beta} \), was obtained by dividing the dose by \((A + B)\), and

The cumulative volume of bile excreted during 1 and 6 h and urine during 6 h in bile-drained animals given 10, 50, 250, and 1000 mg/kg [³H]methotrexate. Data are given as mean ± SD.

Table 1 Cumulative volumes of bile and urine excreted in bile-drained animals

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Dose (mg/kg)</th>
<th>Bile (ml)</th>
<th>Urine (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0–1 h</td>
<td>0–6 h</td>
</tr>
<tr>
<td>A</td>
<td>6</td>
<td>10</td>
<td>1.2 ± 0.1</td>
<td>6.2 ± 1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>1.2 ± 0.4</td>
<td>6.0 ± 1.7</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>250</td>
<td>2.1 ± 0.5</td>
<td>7.3 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>5*</td>
<td>1000</td>
<td>2.0 ± 0.5</td>
<td>9.3 ± 3.1</td>
</tr>
</tbody>
</table>

* \( P < 0.05 \) between 50 and 250 mg/kg.

† Volumes are omitted since bile secretion ceased within 30–90 min in 5 of 6 animals given 1000 mg/kg. One animal that died 30 min after cessation of bile secretion has been excluded.

‡ \( P < 0.05 \) between 250 and 1000 mg/kg.

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 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).
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 (Cl(T)) and biliary clearance (Cl(b)). Animals in group D demonstrated the longest $t_{1/2}$ and largest $V_c$ and $V_f$. 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 (%) 0–2 h</th>
<th>Methotrexate (%) 0–6 h</th>
<th>7-Hydroxy-methotrexate (%) 0–2 h</th>
<th>7-Hydroxy-methotrexate (%) 0–6 h</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>
<td>6.7 ± 3.0</td>
<td>7.3 ± 3.0</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>50</td>
<td>42.2 ± 7.0*</td>
<td>47.8 ± 8.1*</td>
<td>7.3 ± 4.1</td>
<td>8.4 ± 4.8</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>250</td>
<td>25.9 ± 6.3</td>
<td>32.4 ± 9.8</td>
<td>8.3 ± 2.0</td>
<td>9.4 ± 2.4</td>
</tr>
</tbody>
</table>

* P < 0.05 between 50 and 250 mg/kg.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Dose (mg/kg)</th>
<th>Methotrexate (%) 0–6 h</th>
<th>7-Hydroxy-methotrexate (%) 0–6 h</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>B</td>
<td>6</td>
<td>50</td>
<td>23.1 ± 6.0</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>250</td>
<td>30.7 ± 10.8</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.

Fig. 3. Biliary concentrations of methotrexate (○, △, ▽, □, ◊) and 7-hydroxymethotrexate (■, Δ, ▼, ▼, ♦) versus time following a short-time infusion of 1000 mg/kg [3H]methotrexate to 6 anesthetized rats.

MTX administration in 5 of 6 animals at biliary 7-OH-MTX levels of 9800 ± 1100 μM (Fig. 3). In the single rat that secreted bile throughout the experiments a 5-fold lower bile concentration of the metabolite was detected. Furthermore, only the cholestatic animals demonstrated crystalline precipitates in the bile duct when laparotomized at the termination of experiments. Additionally, the bile sampled prior to cessation of bile secretion was found to contain precipitates. Equal amounts of
MTX. In rats infused with 250 mg/kg MTX the maximal rate of transport ($V_{max}$) and the transport constant analogous to the Michaelis–Menten-type kinetics at high dose levels of MTX $K_{MTX}$ was 12 mg/h and 411 $\mu$M (means), respectively, somewhat higher than the findings of Kates and Tozer (61), 12 mg/h and 70 $\mu$M. 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 system following HD-MTX therapy in humans indicates that 7-OH-MTX is implicated in acute hepatotoxicity under the underlying mechanism remains unknown. Our results may in part explain the disproportional increase of peak MTX concentrations in bile and the significant reduction in biliary clearance of MTX, but the underlying mechanism remains unknown. The 7-OH-MTX kinetics deviated compared to the 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.

Figure 5. Serum concentrations of methotrexate (C) and 7-hydroxymethotrexate (B) versus time following short-time infusions of 10 (A), 50 (B), 250 (C), and 1000 (D) mg/kg [3H]methotrexate. Data are given as mean ± SD, n = 6. A single rat of group D that ceased bile secretion within 30 min after MTX administration died 30 min later.

**Table 4 Pharmacokinetic variables in rats given infusions of [3H]methotrexate at four dose levels**

<table>
<thead>
<tr>
<th>Group</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg)</td>
<td>10</td>
<td>50</td>
<td>250</td>
<td>1000</td>
</tr>
<tr>
<td>$t_{1/2}$ (min)</td>
<td>4.2 ± 0.6</td>
<td>4.1 ± 0.9</td>
<td>3.9 ± 1.0</td>
<td>3.9 ± 1.2</td>
</tr>
<tr>
<td>$V_{max}$ (ml/kg)</td>
<td>288 ± 57</td>
<td>245 ± 43</td>
<td>230 ± 42</td>
<td>387 ± 96*</td>
</tr>
<tr>
<td>$V_{m}$ (ml/kg)</td>
<td>776 ± 197*</td>
<td>511 ± 171</td>
<td>417 ± 112</td>
<td>798 ± 138*</td>
</tr>
<tr>
<td>$Cl_{m}$ (ml/min – kg)</td>
<td>13.6 ± 2.9*</td>
<td>10.1 ± 3.8</td>
<td>6.9 ± 1.1</td>
<td>5.0 ± 3.0</td>
</tr>
<tr>
<td>$Cl_{n}$ (ml/min – kg)</td>
<td>3.4 ± 0.4</td>
<td>3.4 ± 0.4</td>
<td>3.4 ± 0.4</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>$Cl_{np}$ (ml/min – kg)</td>
<td>3.7 ± 2.5</td>
<td>2.6 ± 1.7</td>
<td>3.5 ± 0.6</td>
<td>3.1 ± 1.7</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.
* $P < 0.05$ between 10 and 50 mg/kg.
* $P < 0.05$ between 50 and 250 mg/kg.
* $Cl_{np}$ is nonbiliary clearance ($Cl_{np} = Cl_{T} - Cl_{I}$).
of the parent compound showed no signs of saturation, as peak 7-OH-MTX concentrations in bile increased almost proportionally with the dose administered with no reduction in biliary MTX recovery as the 7-hydroxylated metabolite. In comparison to our previous study of bile-drained rats given 10 mg/kg MTX (57), biliary recovery of 7-OH-MTX after this MTX dose was 2-fold larger in the present study. This difference may be due to variations between two different strains of Wistar rats, since the animals used in our previous study were obtained from another source. The 75% larger bile flow within the first hour of the experiments in animals treated with 250 mg/kg MTX may be explained by osmotic choleresis, i.e., increase in bile flow, due to high concentrations of the anionic MTX and 7-OH-MTX moieties present in bile (62, 63).

Despite saturable bile secretion at high serum concentrations of MTX, there were equal nonbiliary clearance and urinary recovery rates of the parent compound at all dosage levels, which corroborate with similar urinary recoveries reported in two previous studies in rats treated with 10 and 250 mg/kg MTX, respectively (57, 64). The significantly higher recovery of 7-OH-MTX in urine after administration of 1000 mg/kg MTX is due to significantly increased serum levels of the metabolite, which may be explained by cholestasis-related cellular injury and the resulting leakage of the hepatic metabolite into sinusoidal blood. The increased urine volume excreted after 1000 mg/kg MTX may result from retention of fluid which would otherwise be eligible for biliary secretion.

Serum MTX elimination in rats given 10 mg/kg was triphasic, which is consistent with previous investigations (57, 64–66). However, the pharmacokinetic variables in all animals were analyzed according to a two-compartment model, since serum MTX disappearance was biphasic after MTX doses surpassing 10 mg/kg. The apparent central volumes of distribution (Vc) and the total clearance (Cl) in animals infused with 10 mg/kg MTX remained unaltered regardless of whether they were analyzed according to a two- or-three-compartment model and were in concordance with previous studies in rats given 1 or 10 mg/kg MTX (57, 67). The experiments gave evidence of a dose-dependent decline in total body clearance (Cl) of MTX, mainly due to a reduction in biliary clearance (Clb) as animals were given larger doses of the drug (Table 4). Central volumes of distribution in rats receiving 10 to 250 mg/kg MTX were, however, equal and consistent with previous studies (57, 67), whereas a significantly larger Vc was found in rats given 1000 mg/kg. The considerably longer terminal MTX half-life, larger Vc, high peak 7-OH-MTX concentration, and long serum half-life of the metabolite after 1000 mg/kg MTX are considered results of cholestasis and the ensuing cessation of drug elimination by bile.

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 leukovorin rescue.

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