Cancer Chemother Pharmacol (1989) 23: 156–160

Cancer Chemotherapy and Pharmacology


# Pharmacokinetics of methotrexate (MTX) and 7-hydroxymethotrexate (7-OH-MTX) in rats and evidence for the metabolism of MTX to 7-OH-MTX

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**Summary.** In pentobarbital anesthetized rats that received 4 mg/kg i.v. methotrexate (MTX) or 7-hydroxymethotrexate (7-OH-MTX), the pharmacokinetics of the two drugs were similar. Plasma concentrations of both drugs declined biexponentially, with terminal half-lives of 90.6 min for MTX and 97.2 min for 7-OH-MTX. The total clearance values were 9.2 and 9.6 ml × kg$^{-1}$ × min$^{-1}$, respectively. With MTX, 48.2% of the dose was excreted in the urine within 200 min and another 31.6% was recovered from the bile; 5.8% was metabolized to 7-OH-MTX and appeared in the bile. Plasma concentrations of the metabolite 7-OH-MTX after MTX administration were below the detection limit. Injected 7-OH-MTX was predominantly excreted into the bile (72.8% of the dose); only 11.2% could be recovered from the urine. Differences between the physicochemical properties of MTX and 7-OH-MTX or different affinities for active transport systems may account for the unequal importance of these two excretion pathways for the two compounds.

## Introduction

The metabolic and elimination pathway of the cytostatic drug methotrexate (MTX) is species-dependent. In rabbits, the greater part of a single MTX dose is excreted in the urine, whereas biliary excretion plays a minor role [5, 17]. Between 15% and 30% of the dose is metabolized to 7-hydroxymethotrexate (7-OH-MTX) [5, 25]; the metabolite is eliminated almost exclusively by the kidneys and its elimination half-life is about 30 min [6, 17]. In man, renal MTX excretion is also of major importance [14, 23], but large concentrations of this drug have also been found in the bile [4, 11]. About 15% of a single high dose is hydroxylated [2], and the half-life of the metabolite is much longer than that of the parent compound [21]. Thus, 7-OH-MTX plasma concentrations may finally exceed MTX concentrations by a factor of 10 or even 100 [7]. In mice and rats, more than 50% of an MTX dose is excreted into the bile [12, 20], and bile duct ligation causes a significant delay in MTX elimination [27]. This prompted us to study biliary MTX elimination in the latter species. As MTX hydroxylation had never been observed in rats before, we were surprised to detect significant amounts of 7-OH-MTX in the bile. Furthermore, our experiments revealed striking differences between the elimination of the parent compound and that of its metabolite.

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## Materials and methods

*Experimental procedure.* Male Wistar rats (270–440 g) were anesthetized by an injection of 60 mg/kg pentobarbital (Nembutal) into a tail vein and placed on their backs on a heated operation table. The body temperature was controlled by a digital thermometer in the rectum and kept constant at 37° C. Additional injections of pentobarbital (10 mg/kg i.v.) were given as needed to maintain anesthesia. A PE 50 catheter was placed in the left arteria carotis and the right vena jugularis. A continuous infusion (8 ml × kg$^{-1}$ × h$^{-1}$) of physiologic saline solution containing 1 mg/ml inulin and 60 mg/ml mannitol was then started. The abdomen was opened by a midline incision and the common bile duct was cannulated with a PE 10 catheter. A double-current polyethylene catheter was sutured into the bladder, allowing the collection of urine and rinsing of the bladder. The abdomen was closed and the urethra was tied by a silk thread at the glans penis to avoid urine loss.

After the surgery was completed, either MTX or 7-OH-MTX (4 mg/kg) was injected as a bolus into the jugular vein. Blood samples (200 µl) were drawn from the carotid artery into heparinized Eppendorf vials at 10, 30, 50, 70, 90, 110, 140, and 180 min and immediately centrifuged. Bile and urine were collected in 20-min intervals for the first 2 h and for two subsequent 40-min periods. The bladder was rinsed with 1 ml physiologic saline at the end of each collection period. Plasma, bile, and urine were stored at −20° C until analysis.

*Analytical procedures.* MTX and 7-OH-MTX in plasma, bile, and urine were measured by HPLC as previously described in detail [17]. The detection limit for both compounds was 0.1 µg/ml. Inulin in plasma and urine was determined with anthrone reagent [10].

*Drugs.* A commercially available preparation of MTX (Methotrexat-Lederle; Cyanamid-Lederle, Wolfratshausen, FRG) was used. 7-OH-MTX was prepared by the method of Cairnes and Evans [3], using aldehyde oxydase from homogenized rabbit liver. 7-OH-MTX was precipitated from the reaction buffer and purified by chromatography on an XAD column. The 7-OH-MTX finally obtain-

ed was 90% pure and contained no detectable amounts of MTX. Neither HPLC – the compound eluted from the column as a single peak – nor the UV spectrum gave evidence of contamination with other absorbing material.

*Calculations and statistics.* A biexponential function was fitted to the plasma concentration-time curve of MTX or 7-OH-MTX in each rat, by successive curve peeling. The usual formulae [9] were then used for the calculation of the pharmacokinetic parameters. Geometric means from seven experiments and their 95% confidence limits are given in the figures and tables. Bile or urine flow as well as renal or biliary clearance values from consecutive collection periods were compared by Student's *t*-test for paired data. $P \leq 0.05$ (two-tailed) was set as the threshold for statistical significance.

## Results

### *MTX kinetics*

MTX plasma concentration declined biexponentially after i.v. injection of this drug (Fig. 1). In seven animals, the mean half-lives of the α and β phases were 12.5 and 90.6 min, respectively, and the mean total body clearance was 9.2 ml × kg$^{-1}$ × min$^{-1}$. The pharmacokinetic parameters and their 95% confidence limits are listed in Table 1. MTX was excreted in the urine and bile (Fig. 1). After 200 min, 48.3% (range, 41.8%–55.7%) of the injected dose had appeared in the urine. The greatest mean renal MTX clearance (6.1 ml × kg$^{-1}$ × min$^{-1}$) was calculated for the second 20-min collection period, and the subsequent decline to 2.9 ml × kg$^{-1}$ × min$^{-1}$ was statistically significant (Table 2).

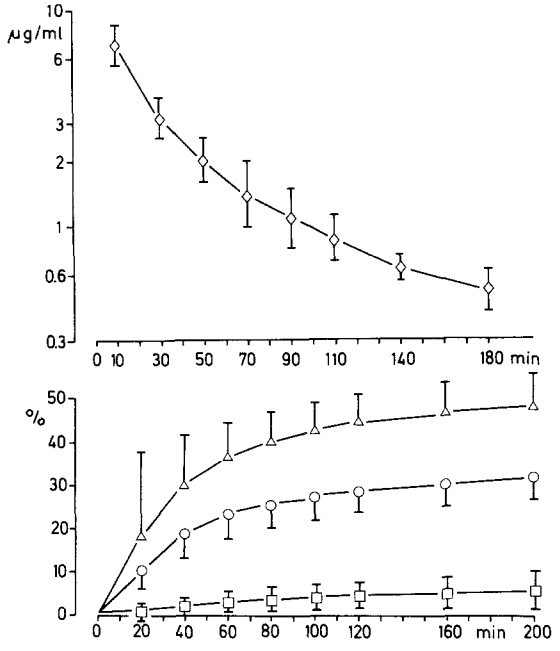


**Fig. 1.** Plasma concentration (*upper panel*) and cumulated excretion (*lower panel*) of MTX after i.v. injection of 4 mg/kg. ◇, MTX plasma concentration; △, MTX excretion in urine; □, 7-OH-MTX excretion in bile; ○, MTX excretion in bile. Symbols indicate the geometric means and their 95% confidence limits ($n = 7$). Abscissae: time after drug injection (min). Ordinates: drug concentration (µg/ml) or cumulated drug excretion (percentage of dose)

**Table 1.** Pharmacokinetic parameters of MTX and 7-OH-MTX after i.v. bolus injection of 4 mg/kg. Geometric means and their 95% confidence limits are shown ($n = 7$)

| Parameter | | MTX | 7-OH-MTX |
|---|---|---|---|
| A | (µg × ml$^{-1}$) | 8.2<br>5.3–12.7 | 6.9<br>5.1–9.1 |
| B | (µg × ml$^{-1}$) | 2.1<br>1.3–3.4 | 1.8<br>1.2–2.5 |
| $t_{0.5\alpha}$ | (min) | 12.5<br>9.5–16.4 | 15.9<br>13.6–18.6 |
| $t_{0.5\beta}$ | (min) | 90.6<br>63.8–128.7 | 97.2<br>79.9–118.6 |
| $V_c$ | (ml × kg$^{-1}$) | 371.6<br>279.0–494.9 | 453.2<br>377.5–544.1 |
| $V_p$ | (ml × kg$^{-1}$) | 420.2<br>240.2–734.9 | 424.1<br>314.8–571.4 |
| $K_{10}$ | (min$^{-1}$) | 0.025<br>0.018–0.035 | 0.021<br>0.015–0.028 |
| $Q_{TOT}$ | (ml × kg$^{-1}$ × min$^{-1}$) | 9.2<br>8.6–9.9 | 9.6<br>7.6–12.0 |

*Abbreviations: A, B,* initial concentrations for α and β-phases; $t_{0.5\alpha}$, $t_{0.5\beta}$, half-lives of α and β phases; $V_c$, $V_p$, volumes of the central and peripheral compartments; $K_{10}$, elimination constant; $Q_{TOT}$, total body clearance

Only small variations in renal inulin clearance were observed (Table 2), whereas the total urine flow increased from 5.0 (range, 3.9–6.4) to a maximum of 7.1 ml × kg$^{-1}$ × h$^{-1}$ (range 5.7–8.9) at 60–80 min after MTX injection, declining back to 5.7 ml × kg$^{-1}$ × h$^{-1}$ (range 4.4–7.3) at the end of the experiments. The mean ratio of renal MTX-to-inulin clearance ranged between 2.5 and 4.5 in the different collection periods, indicating active tubular secretion of MTX.

On average, 31.6% (range, 26.7%–37.3%) of the MTX dose was recovered from the bile in unchanged form (Fig. 1). The mean bile flow varied between 2.1 and 2.6 ml × kg$^{-1}$ × h$^{-1}$, whereas the biliary MTX clearance dropped from a peak value of 4.9 (second collection period) to 2.5 ml × kg$^{-1}$ × min$^{-1}$ at the end of the observation period (Table 2). An average of 5.7% (range, 3.2%–10.3%) of the MTX dose was metabolized to 7-OH-MTX and secreted into the bile. The 7-OH-MTX metabolite concentrations in plasma were too low to be measured (i.e., < 0.1 µg/ml), although peaks indicating very small quantities of the metabolite were detectable in chromatograms from two animals; thus, 7-OH-MTX metabolite biliary clearance could not be calculated. The mean concentration ratio of 7-OH-MTX to MTX in bile was 0.1 during the first collection period, increasing steadily to a final value of 0.4.

### *7-OH-MTX kinetics*

The kinetics of 7-OH-MTX after its i.v. injection resembled MTX kinetics (Fig. 2). The mean half-lives of the α and β phases (15.9 and 97.2 min, respectively), total body clearance (9.6 ml × kg$^{-1}$ × min$^{-1}$), and other pharmacokinetic parameters showed no significant differences (Table 1). However, 7-OH-MTX was mainly excreted into the bile;

**Table 2.** Renal and biliary clearance values (ml × kg$^{-1}$ × min$^{-1}$) for MTX and 7-OH-MTX during the consecutive collection periods. Geometric means and their 95% confidence limits are shown ($n = 7$)

| Drug | | 0–20 min | 20–40 min | 40–60 min | 60–80 min | 80–100 min | 100–120 min | 120–160 min | 160–200 min |
|---|---|---|---|---|---|---|---|---|---|
| MTX 4 mg/kg i.v. | $Q_{BIL}$ | 3.0<br>1.7–5.3 | 4.9<br>3.4–7.1 | 3.8<br>3.2–4.6 | 3.3<br>2.7–4.1 | 3.1<br>2.5–3.8 | 2.1<br>1.5–3.1 | 2.7<br>1.9–3.8 | 2.5<br>1.6–3.9 |
| | $Q_{REN}$ | 5.2<br>2.6–10.5 | 6.1<br>5.4–6.9 | 5.3<br>3.8–7.4 | 4.3<br>2.8–6.5 | 4.3<br>3.0–6.1 | 3.6<br>2.5–5.0 | 3.0<br>1.5–6.0 | 2.9<br>1.5–5.4 |
| | $Q_{INU}$ | 1.2<br>0.8–2.0 | 1.4<br>1.0–1.8 | 1.3<br>1.0–1.7 | 1.2<br>0.9–1.5 | 1.1<br>0.8–1.6 | 1.2<br>0.9–1.5 | 1.2<br>0.9–1.5 | 1.1<br>0.9–1.5 |
| 7-OH-MTX 4 mg/kg i.v. | $Q_{BIL}$ | 9.6<br>7.8–11.9 | 10.0<br>7.8–12.7 | 8.5<br>6.1–11.8 | 7.5<br>4.6–12.3 | 7.7<br>6.0–9.9 | 6.3<br>4.9–8.3 | 5.4<br>3.9–7.4 | 5.8<br>5.0–6.8 |
| | $Q_{REN}$ | 1.4<br>1.1–1.9 | 1.5<br>1.1–1.9 | 1.3<br>0.9–1.9 | 1.5<br>1.1–2.0 | 1.5<br>1.0–2.2 | 1.0<br>0.4–2.4 | 0.5<br>0.2–1.2 | 0.3<br>0.1–1.4 |
| | $Q_{INU}$ | 1.3<br>1.0–1.7 | 1.3<br>0.8–2.0 | 1.3<br>0.9–1.8 | 1.4<br>1.0–2.1 | 1.5<br>1.0–2.3 | 1.4<br>0.9–2.0 | 1.1<br>0.6–2.0 | 1.24<br>0.7–2.1 |

*Abbreviations:* $Q_{BIL}$, biliary clearance; $Q_{REN}$, renal clearance; $Q_{INU}$, renal inulin clearance

72.8% (range, 65.2–81.3%) of the injected dose appeared within 200 min (Fig. 2). The mean bile flow varied between 2.9 and 3.7 ml × kg$^{-1}$ × h$^{-1}$, and the mean biliary 7-OH-MTX clearance dropped significiantly from 10.0 ml × kg$^{-1}$ × min$^{-1}$ during the second collection period to 5.8 ml × kg$^{-1}$ × min$^{-1}$ at the end of the experiments (Table 2). Only 11.2% (range, 8.0%–15.8%) of the 7-OH-MTX dose was recovered from the urine (Fig. 2). Whereas renal inulin clearance remained nearly stable, renal 7-OH-MTX clearance decreased drastically from 1.5 to 0.3 ml × kg$^{-1}$ × min$^{-1}$ (Table 2); thus, the mean ratio of 7-OH-MTX-to-inulin clearance dropped from 1.1 to 0.2. The mean urinary flow ranged between 6.3 (range, 4.5–9.1) and 5.1 ml × kg$^{-1}$ × h$^{-1}$ (range, 4.0–6.5) in the different collection periods.

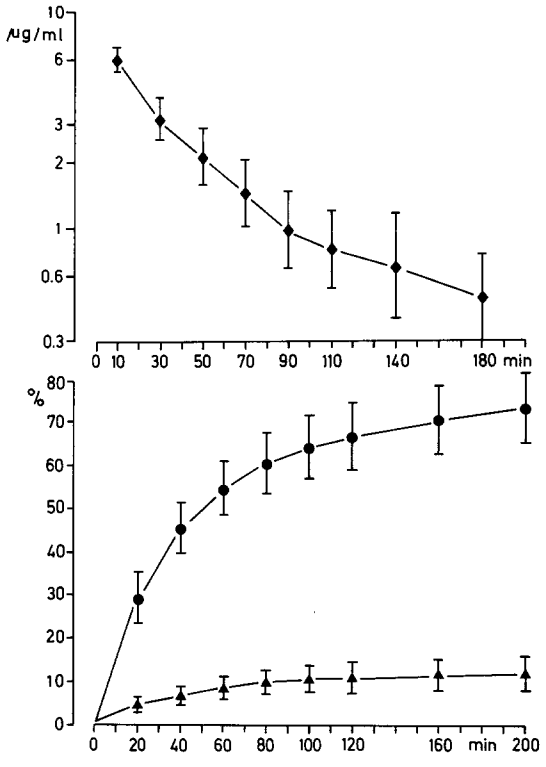


**Fig. 2.** Plasma concentration (*upper panel*) and cumulated excretion (*lower panel*) of 7-OH-MTX after i.v. injection of 4 mg/kg. ◆, 7-OH-MTX plasma concentration; ▲, 7-OH-MTX excretion in urine; ●, 7-OH-MTX excretion in bile. Symbols indicate the geometric means and their 95% confidence limits ($n = 7$). Abscissae: time after drug injection (min). Ordinates: drug concentration (µg/ml) or cumulated drug excretion (percentage of dose)

## Discussion

After injecting wakeful rats with 3.1 mg/kg MTX Scheufler [26] described a biexponential decline in the plasma concentration of this drug with half-lives of 10.6 (α phase) and 50.1 min (β phase) and a total body clearance of 13.5 ml × kg$^{-1}$ × min$^{-1}$. The anesthesia and surgery carried out on our animals may explain the slower drug elimination in the present experiments. Pentobarbital anesthesia is known to decrease the glomerular filtration rate [28], which may explain why the inulyn clearance was lower than usually reported in the literature (2.9–6.5 ml × kg$^{-1}$ × min$^{-1}$) [1]. A renal MTX clearance well above the inulin clearance indicates that in rats as in other species [13, 15, 22], this drug is excreted by active tubular secretion. Additional tubular reabsorption, known to occur in dogs [13] and rabbits [16] may also occur. At least the existence of a reuptake system with low capacity, which is saturated at the beginning of the experiments and reabsorbs an increasing fraction as the MTX plasma concentration declines, would explain the continuous decrease in renal MTX clearance. However, this explanation is not unequivocal, because an increase in the fraction of MTX bound to plasma proteins could have a similar effect. An osmotic effect of the drug may be responsible for the transient increase in the urinary flow after MTX injection.

As far as we know, our detection of 7-OH-MTX in rat bile is the first indication of hydroxylation of MTX in this species. In a previous study on biliary elimination of MTX in rats [12], the authors used tritiated MTX and their technique did not distinguish between the parent compound and its metabolite. We could not measure 7-OH-MTX concentrations in rat plasma. This confirms the results of two earlier investigations [19, 29], which demonstrated that on-

ly small amounts of some unidentified metabolites thought to be produced by intestinal bacteria appear in rat plasma, but not 7-OH-MTX. It seems that MTX is hydroxylated in the rat liver and secreted directly into the bile. Therefore, one would expect a constant ratio of 7-OH-MTX-to-MTX concentration in the bile, provided that the metabolism is not dose-dependent. Instead, the ratio increased continuously during the observation period. Thus, the metabolizing enzyme seems to be saturated by the high MTX concentrations immediately after drug injection, whereas with decreasing plasma concentration an increasing fraction can be hydroxylated. Indeed, recent in vitro experiments [24] have shown that rat hepatocytes can produce 7-OH-MTX from MTX. However, the metabolizing capacity of rat liver aldehyde oxydase is very low (about 0.5%) compared with that of rabbit liver enzyme [18].

In contrast to MTX, i.v. injected 7-OH-MTX appeared mainly in the bile. The fact that the total bile flow was somewhat greater in 7-OH-MTX-injected animals while MTX produced a greater diuretic effect may be ascribed to an osmotic effect of the two drugs. Tubular secretion cannot contribute very much to renal 7-OH-MTX elimination, because initially the renal drug clearance was not higher than inulin clearance. However, tubular reabsorption could explain the final drop in 7-OH-MTX clearance. Again, the transport capacity would have to be rather small, being saturated during the first half of the experiments.

It is unlikely that the impurities in the 7-OH-MTX preparation (10%; see *Materials and methods*) are responsible for the limited renal excretion of 7-OH-MTX because in a previous study done in rabbits [16], with the same batch of 7-OH-MTX, no influence on the renal elimination of either 7-OH-MTX or MTX was observed. Why, then, is 7-OH-MTX eliminated mainly into the bile? The metabolite is less water-soluble but more lipophilic than the parent compound; these physicochemical properties should favor the biliary elimination route. In addition, the difference between the molecular weights of the two compounds might play a role. In the rat, water-soluble drugs with a molecular weight of up to 200 daltons are excreted by the kidneys. With increasing size of the molecule, biliary excretion becomes more important and compounds with molecular weights $>465$ daltons appear almost exclusively in the bile [8]. The molecular weight of MTX (454.5 daltons) is just below the upper limit for renal elimination, whereas that of 7-OH-MTX (470 daltons) slightly exceeds this threshold, thus favoring biliary secretion of the latter. Furthermore, hydroxylation of MTX to 7-OH-MTX could increase the affinity for an active transport system into the bile. Species differences in this mechanism seem to be an attractive explanation for the much greater importance of biliary excretion of 7-OH-MTX in rats as compared with rabbits [5]. However, at the moment none of these hypothetical explanations can be supported by clear-cut experimental data.

In summary, the detection of 7-OH-MTX as a metabolite of MTX in the rat and the observation of significant differences in the elimination of both compounds have added interesting new aspects to the pharmacokinetics of MTX.

*Acknowledgements.* The authors wish to thank Mrs. M.-L. Stolte for careful preparation of the figures and Mrs. G. Mundhenke for typing the manuscript.

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Received February 12, 1988/Accepted August 5, 1988