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Pharmacokinetics of methotrexate and 7-hydroxy-methotrexate in rabbits

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Summary. In rabbits the IV kinetics of MTX (1.33, 4 and 12 mg/kg) could be described by a linear three-compartment model with a terminal half-life between 2.4 and 3.6 h. During 8 h 50% of the dose was excreted into urine in unchanged form and 15% as the metabolite 7-OH-MTX. These fractions remained constant with increasing dose. In continuous infusion experiments (9–900 μg/kg × min MTX IV) a decrease of the renal MTX clearance with increasing plasma concentration was observed. This effect was nearly compensated by an increase of the extrarenal MTX clearance. After short-term infusion of 7-OH-MTX (4 mg/kg) a biexponential decline of 7-OH-MTX plasma concentrations was observed with a terminal half-life of 0.45 h. About 80% of the dose was regained from urine during 5 h. From the combined pharmacokinetic data a linear model was constructed for the calculation of 7-OH-MTX plasma concentrations after short-term MTX infusion. For the first 4 h after MTX application the predicted values were in good accordance with the 7-OH-MTX concentrations actually measured.

Introduction

In recent years interest has focused on 7-hydroxymethotrexate (7-OH-MTX), a metabolite of methotrexate (MTX). In man 7-OH-MTX plasma concentrations decline very slowly and 24–48 h after high-dose MTX infusion plasma concentrations of the metabolite are often more than 10 times as high as those of the parent compound [4, 15]. 7-OH-MTX has no known therapeutic effect, but owing to its poor aqueous solubility it may crystallize in the kidney and thus contribute to the nephrotoxicity of MTX infusions [13]. Also, the parent drug and the metabolite may compete for the same active renal excretion mechanism. To get detailed informations on the pharmacokinetics of 7-OH-MTX a direct application of this metabolite in an appropriate animal model would be necessary. We thought that rabbits could be suitable for these experiments because (a) they are easy to handle and allow bladder catheterization and repeated blood sampling and (b) they are known to produce large amounts of 7-OH-MTX from MTX [5, 22]. In the present paper we report detailed data on the pharmacokinetics and urinary excretion of MTX and 7-OH-MTX after short-term IV infusion and during continuous IV application. A simple, linear pharmacokinetic model is then constructed to predict the plasma concentrations of 7-OH-MTX after IV application of MTX.

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Materials and methods

1. Experimental procedures. Nineteen male rabbits weighing 3.6–5.5 kg were used in the study. They were housed individually and had free access to Altromin standard diet and tap water.

The dose-dependence of IV kinetics of MTX was assessed in six animals. A dose of 1.33, 4 or 12 mg/kg MTX dissolved in physiological saline solution (10 ml/kg) was infused over 10 min into an ear vein by a Braun Infusomat (Braun, Melsungen; FRG). An interval of 4 weeks was allowed between doses. Blood samples of approximately 2 ml were drawn from the contralateral ear artery at the end of the infusion and 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 h later. Urine was collected by catheterization with an 8-F rubber catheter before and 2, 4, and 8 h after the end of infusion. Any urine voided spontaneously between these collection times was added to the appropriate fraction.

Another seven rabbits were used for the investigation of the kinetics of 7-OH-MTX following IV administration. They received 4 mg/kg 7-OH-MTX dissolved in 0.9% NaCl solution (2 ml/kg) by infusion over 2 min. Blood samples were drawn at the end of infusion and at appropriate time intervals. In four animals the bladder was catheterized by means of an indwelling 8-F Foley balloon catheter for continuous sampling of urine.

To study steady-state kinetics, six further animals received MTX by continuous infusion. These rabbits were kept lightly anesthetized with an initial dose of 30 mg/kg pentobarbital (Nembutal®) IV and additional doses of 2–3 mg/kg every 10–15 min. The first series of experiments began with an IV bolus injection of 0.15 mg/kg MTX and an infusion of 9 μg/kg × min MTX in a volume of 0.1 ml/kg × min for 80 min. Then an additional IV bolus of 0.3 mg/kg MTX was injected and the infusion rate was raised to 27 μg/kg × min by adjusting the MTX concentration for another 80 min. A third bolus injection of MTX (1.05 mg/kg) and an infusion of 90 μg/kg × min for a final 80-min period finished the experiment. Blood samples were drawn every 10 min during the infusion period and urine was collected at 20-min intervals from an indwelling catheter. At the end of each collection period the bladder was carefully rinsed with 10 ml saline. The

rabbits were allowed to recover from anesthesia and 4 weeks later a similar study was performed on the same animals, this time with consecutive infusion rates of 90, 270, and 900 µg/kg × min with associated bolus injections of 1.5, 3 and 10.5 mg/kg MTX.

Blood samples were centrifuged immediately for 2 min in an Eppendorf 3200 centrifuge, and plasma and urine were stored at −20 °C until analysis.

2. Analytical procedures. MTX concentrations in plasma were measured with an enzymatic assay [23]. 7-OH-MTX in plasma was determined by HPLC. To precipitate proteins 0.2 ml 0.04 N acetic acid was added to 0.4 ml plasma, whereupon the mixture was heated for 5 min at 96 °C and then centrifuged for 2 min at 8000 *g*. Then 100 µl of the clear supernatant was injected onto a 0.4 × 25 cm Shandon ODS 5-µm column. The column was perfused with a solvent mixture (v:v) of 18% methanol, 2% acetonitril in 0.1 *M* phosphate buffer, pH 7.0, at a rate of 1.2 ml/min and the eluate monitored at 308 nm. In urine MTX and 7-OH-MTX were determined by HPLC.

3. Drugs and reagents. MTX was used as commercially obtained from Lederle (Wolfratshausen). 7-OH-MTX was prepared with enzyme from rabbit liver as described elsewhere [14]. The preparation was found to be 72% pure on UV absorption. MTX contamination was not detectable (HPLC, lower limit of sensitivity: less than 0.1% MTX in 7-OH-MTX). All other chemicals were of analytical grade and were obtained from Merck, Darmstadt.

4. Calculations and statistics. The values given are geometric means and their 95% confidence limits. Bi- or triexponential functions were fitted to the plasma concentrations of MTX and 7-OH-MTX after short-term infusion by the method of successive curve peeling. Pharmacokinetic parameters were calculated with allowance for the infusion time [19].

In the experiments with continuous infusion of MTX, the total clearance of MTX was calculated by dividing the infusion rate (µg/kg × min) by the mean plasma concentration (µg/ml) during the last 20 min of each infusion period. The renal clearances of MTX and 7-OH-MTX were calculated by dividing the amount excreted into the urine (µg) by the duration of the collection time (min), the corresponding mean plasma concentration (µg/ml), and the weight of the animals (kg). The clearance values of the six different infusion periods were compared by Student's *t*-test for paired data ($P \leq 0.05$; two-tailed) to assess statistically significant differences.

The pharmacokinetic model used to predict the plasma concentrations of 7-OH-MTX after short-term infusion of MTX is described in detail under *Results*. The method of Laplace transforms [9] was used to find the pentaexponential function that describes the plasma concentration of 7-OH-MTX.

Results

1. Short-term infusion of MTX and 7-OH-MTX

The plasma concentrations of MTX during the first 12 h after infusion of 12, 4, and 1.33 mg/kg are shown in Fig. 1. An almost parallel decline of drug concentrations was seen with the three doses. Triexponential curves were fitted to the data, and pharmacokinetic parameters were calculated assuming a three-compartment mamillary model with elimination from the central compartment. The results are given in Table 1. With linear kinetics the AUC (AUC = area under the curve) would be expected to increase by a factor of 3 between doses. The observed values come close to this prediction, and there is no systematic variation of the total body clearance or the renal drug excretion with the administered dose. The increase of the terminal half-life with increasing drug dose does not necessarily indicate nonlinear kinetics. The 24h values for

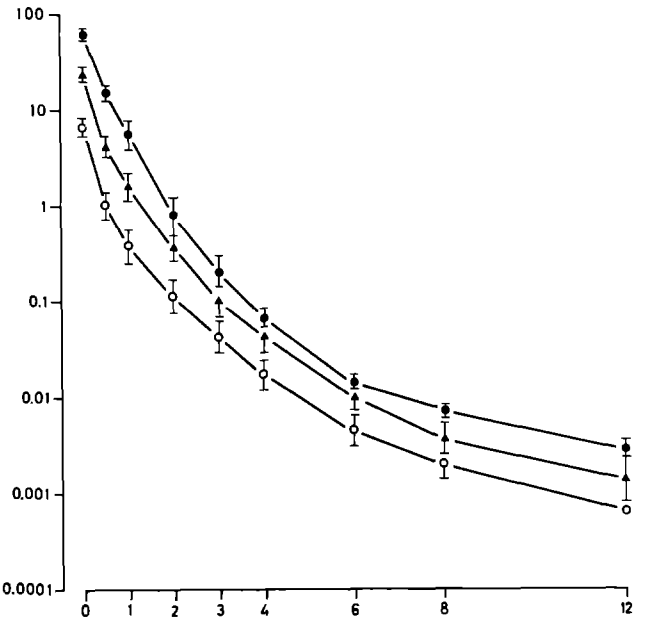

Fig. 1. Plasma levels of MTX after infusion of 1.33 (○), 4 (▲), and 12 (●) mg/kg during 10 min. The *symbols* indicate geometric means and their 95% confidence limits ($n=6$). *Abscissa*, time after the end of infusion (h); *ordinate*, MTX concentration (µg/ml)

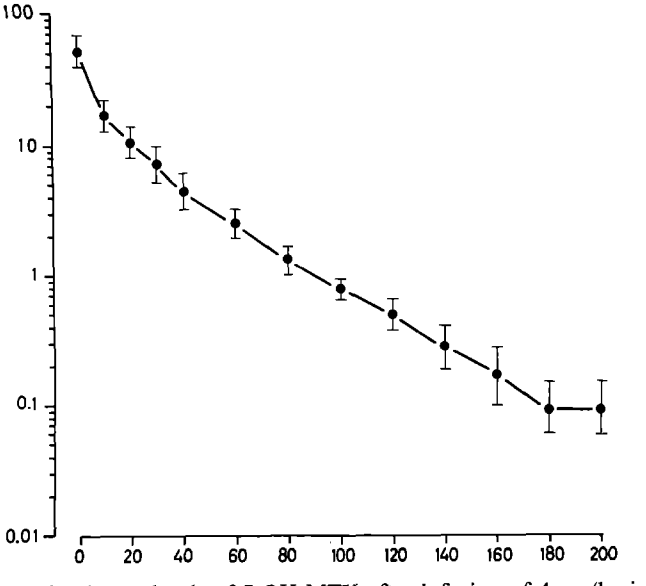

Fig. 2. Plasma levels of 7-OH-MTX after infusion of 4 mg/kg in 2 min. *Symbols* indicate geometric means and their 95% confidence limits ($n=7$). *Abscissa*, time after the end of infusion (min); *ordinate*, drug concentration (µg/ml)

Table 1. Pharmacokinetic parameters of MTX and 7-OH-MTX as calculated from the mean plasma concentrations ($n=6$ or 7) after short-term infusion.

		MTX			7-OH-MTX
		1.33 mg/kg	4 mg/kg	12 mg/kg	4 mg/kg
	A	5.908	20.07	49.27	30.97
	B	0.927	4.459	12.21	10.69
	C	0.0204	0.0282	0.0286	
Half-lives (h)	$t_{0.5\alpha}$	0.136	0.146	0.206	0.109
	$t_{0.5\beta}$	0.632	0.553	0.507	0.448
	$t_{0.5\gamma}$	2.402	2.739	3.642	
Volumes of distribution (ml/kg)	V_c	136	118	154	88
	V_{II}	89	57	30	51
	V_{III}	32	13	9	
	V_β	1696	1553	2154	207
	V_{ss}	257	188	193	139
Total body clearance (ml/kg × min)	Q_{TOT}	8.15	6.55	6.83	5.33
Area under the curve (h · µg/ml)	AUC	2.72	10.18	29.27	12.5
Renal drug excretion[a] (% of dose)	As MTX	27.7 (11.9–64.5)	48.8 (28.9–82.2)	49.5 (38.0–64.5)	
	As 7-OH-MTX	17.5 (11.7–26.1)	13.5 (9.2–19.8)	15.5 (10.1–23.9)	78.9 (71.6–87.0)
	Total[b]	47.6 (24.4–92.93)	63.1 (39.1–102.0)	65.7 (50.6–85.3)	

[a] During an observation period of 8 h in the case of MTX and 5 h in the case of 7-OH-MTX

[b] Note that geometric means were calculated; thus "Total" will not give the arithmetic total of the two fractions

The renal excretion was calculated from the amount of drug recovered from the urine. For this parameter geometric means and their 95% confidence limits are listed. In the case of MTX, the amount of drug excreted in unchanged form and the amount metabolized to 7-OH-MTX have been assessed separately

MTX concentrations could be measured for the largest but not for the two lower doses. This shortcoming of the method may have caused underestimation of the γ-phase in the latter cases. A maximum of 66% of the dose was recovered from urine during 8 h, about 50% in unchanged form and 15% as the metabolite 7-OH-MTX. As a total elimination of more than 99% was calculated from the AUC, additional unidentified sites of loss must be present.

Reliable pharmacokinetic data for a metabolite cannot usually be obtained from infusion experiments with the parent drug. Therefore, 7-OH-MTX itself was infused (4 mg/kg in 2 min) in seven rabbits. The resulting mean plasma concentrations are shown in Fig. 2. At 200 min after the end of infusion the 7-OH-MTX concentration was near the detection limit of the HPLC method. A biexponential curve was fitted to the data and the parameters of the corresponding two-compartment open model with central elimination are included in Table 1. In the four catheterized animals 78.9% of the dose was recovered from the urine after 5 h, whereas an excretion of more than 99% is predicted from the AUC.

2. Continuous infusion of MTX

The combination of bolus injections and continuous MTX infusions established constant plasma concentrations of MTX and 7-OH-MTX during the last 20 min of each 80-min infusion period (Fig. 3) and clearance values for both drugs could be calculated (Table 2). The stepwise increase of the infusion rate by a factor of 3 (27/9) or 3.33 (90/27) produced a similar increase in the steady-state plasma con-

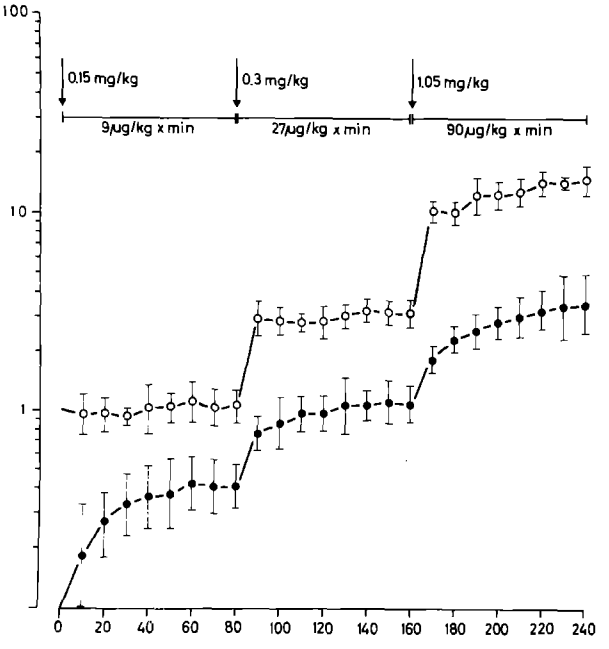

Fig. 3. Plasma levels of MTX (○) and 7-OH-MTX (●) during continuous infusion of MTX. The *symbols* indicate geometric means and their 95% confidence limits. The infusion rate was increased stepwise from 9 to 90 µg/kg × min as indicated above the figure. Bolus injections of 0.15, 0.3, and 1.05 mg/kg MTX were given as indicated to achieve constant plasma concentrations more quickly. *Abscissa*, time after beginning of the infusion (min); *ordinate*, drug concentration (µg/ml)

Table 2. Plasma concentrations and clearance values of MTX and 7-OH-MTX during continuous infusion of MTX. Plasma concentrations during the last 20 min of each infusion period were used for clearance calculations. Geometric means and their 95% confidence limits ($n=6$) are given for all parameters. Successive infusions of 9, 27 and 90 μg/kg × min were given to the animals during the first experiment *(upper part)* whereas the infusions of 90, 270, and 900 μg/kg per min *(lower part)* were administered to the same animals 4 weeks later, as described under *Methods*. For the clearance data, the asterisks indicate means that are significantly different from the corresponding value of the first infusion period (9 μg/kg × min) according to Student's *t*-test for paired data ($P<0.05$)

	Plasma concentrations (μg/ml)		Clearance values (ml/kg × min)		
(μg/kg × min)	C_{MTX}	C_{70H}	$Q_{TOT/MTX}$	$Q_{REN/MTX}$	$Q_{REN/70H}$
9	1.07	0.42	8.39	4.61	4.04
	0.88–1.31	0.31–0.55	6.86–10.26	3.54–6.01	2.32–7.02
27	3.15	1.08	8.59	4.42	5.04
	2.79–3.56	0.91–1.29	7.56–9.77	3.56–5.47	3.03–8.38
90	14.14	3.43	6.43*	3.75	5.04
	12.51–15.98	2.66–4.43	5.56–7.44	2.85–4.95	3.41–7.46
90	15.58	5.36	5.78*	3.03*	3.17
	13.40–18.12	4.42–6.50	4.97–6.72	2.26–4.07	2.53–3.98
270	41.65	12.80	6.48	2.48*	3.27
	32.15–53.96	10.40–15.70	5.00–8.40	1.85–3.32	2.79–3.83
900	192.64	49.99	4.67	1.31*	1.57*
	125.05–296.77	31.75–78.73	3.03–7.10	0.79–2.18	0.72–3.41

centrations of MTX and 7-OH-MTX. The ratio of the MTX to 7-OH-MTX concentration at the end of each infusion period remained roughly constant. The total clearance of MTX was well in keeping with the values obtained from the IV kinetics (6.5–8.1 ml/kg × min). Though some decrease was observed with increasing plasma concentration, this effect was not very impressive, except for the largest plasma concentration. The decrease of the renal MTX clearance with increasing plasma concentration was much more prominent. This parameter was reduced to about 30% of its initial value with the largest infusion rate while the amount of MTX excreted into urine during steady state decreased from 55% to 25% of the infused dose. The renal clearance of 7-OH-MTX was slightly lower than its total clearance as determined from the short-term infusion (5.3 ml/kg × min) and showed only small variations with increasing plasma concentration. A nearly constant fraction (15%–18%) of the infused MTX was regained from the urine as the metabolite.

3. Model calculations to predict 7-OH-MTX plasma concentrations after short-term MTX infusion.

If linear pharmacokinetics of MTX and 7-OH-MTX are assumed (see *Discussion*) the plasma concentrations of 7-OH-MTX after short-term infusion of MTX can be calculated. The pharmacokinetic model is shown in Fig. 4. MTX is infused with the zero-order infusion constant K_o into the central compartment ($V_{C/MTX}$). It is distributed to two peripheral compartments (V_{II} and V_{III}). The intercompartmental transfer constants can be derived from the IV kinetics. The elimination constant K_{10} is split into a formation constant K_f, which characterizes the metabolization to 7-OH-MTX, and a residual elimination constant K_{el} for renal and unidentified nonrenal elimination. 7-OH-MTX is distributed between two compartments ($V_{C/70H}$ and V_V) and is finally eliminated. The rate constants K_{45}, K_{54}, and K_{40} may again be taken from the 7-OH-MTX IV kinetics. Thus, for a full description of the model, only K_f and K_{el}

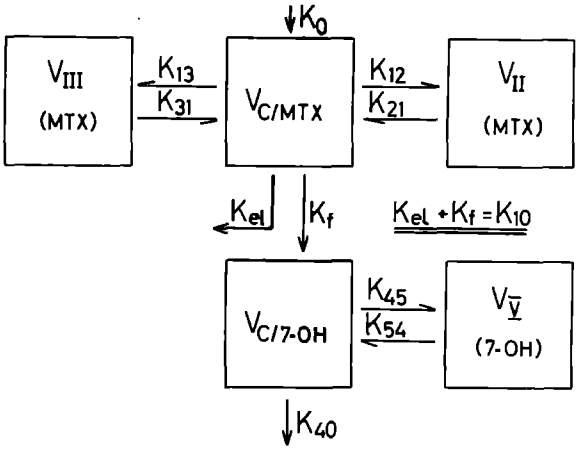

Fig. 4. Schematic representation of the pharmacokinetic model used to predict the plasma concentrations of 7-OH-MTX after infusion of MTX. MTX is applied to a central compartment ($V_{C/MTX}$) and distributes to two peripheral compartments (V_{II}, V_{III}). A part of the dose is metabolized to 7-OH-MTX with the rate constant K_f. The metabolite distributes between a central ($V_{C/70H}$) and a peripheral compartment (V_v). Plasma concentrations of 7-OH-MTX must therefore be described by a pentaexponential function

need to be calculated. This can be done from the concentration ratio of MTX and 7-OH-MTX in the continuous infusion experiments:

$$K_f = K_{40} \cdot \frac{C_{70H} \cdot V_{C/70H} \cdot MW_{MTX}}{C_{MTX} \cdot V_{C/MTX} \cdot MW_{70H}}; \quad K_{el} = K_{10} - K_f.$$

In this formula C_{70H} and C_{MTX} are the plasma concentrations of the two drugs during steady state; $V_{C/70H}$ and $V_{C/MTX}$, the volumes of the corresponding central compartments; and MW_{MTX} and MW_{70H}, the molecular weights. The plasma concentration of MTX after the infusion of 12 mg/kg in 10 min is described by the equation (kinetic data from Table 1):

$$C_{MTX} = 39.27 \cdot e^{-3.366t} + 12.21 \cdot e^{-1.366t} + 0.0286 \cdot e^{-0.1903t}.$$

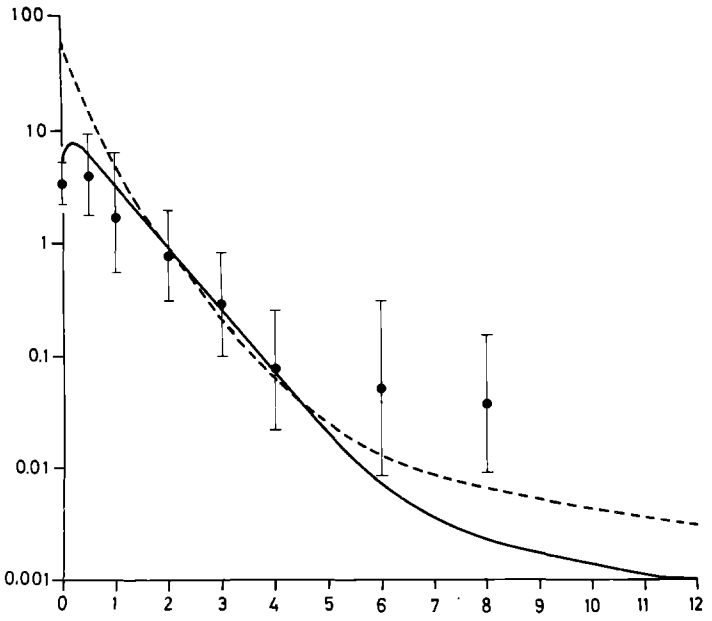

Fig. 5. Plasma concentrations of MTX *(broken line)* and 7-OH-MTX *(solid line)* as predicted by the pharmacokinetic model (Fig. 4). An infusion of 12 mg/kg MTX in 10 min was assumed and rate constants and distribution volumes shown in Table 1 were used for the calculations. The concentrations of 7-OH-MTX (geometric means and their 95% confidence limits; $n=6$) measured after application of 12 mg/kg MTX are also shown. *Abscissa,* time after the end of infusion (h); *ordinate,* drug concentration (μg/ml)

For 7-OH-MTX a formation constant $K_f = 0.6104 \times h^{-1}$ was calculated and the remaining kinetic data from Table 1 were used to derive the following pentaexponential function for the description of the 7-OH-MTX plasma concentrations after MTX infusion (12 mg/kg):

$$C_{70H} = 6.32 \cdot e^{-3.366t} + 19.37 \cdot e^{-1.366t} + 0.0091 \cdot e^{-0.1930t} - 12.08 \cdot e^{-6.359t} - 9.15 \cdot e^{-1.547t}.$$

These two curves are shown in Fig. 5. The plasma concentrations of 7-OH-MTX actually measured after the infusion of 12 mg/kg MTX are also shown. As can be seen, there is a reasonable agreement between the predicted and the measured concentrations at least during the first 4 h. After infusion of 4 mg/kg MTX a similar agreement was found (data not shown), whereas 7-OH-MTX concentrations after administration of 1.33 mg/kg MTX were too low to be quantified.

Discussion

We obtained a triexponential decline of MTX plasma concentrations after IV injection, which is in line with observations [2, 25, 28] in other animal species. In our short-term infusion experiments the AUC increased in proportion with increasing dose, and the other kinetic parameters showed no systematic variation. Thus the IV kinetics of MTX in the tested dose range could be described by a linear model. In our continuous infusion experiments the total body clearance of MTX remained nearly constant and the plasma concentration was linearly correlated to the infusion rate. However, the renal MTX clearance decreased with increasing plasma concentration. This corresponds to the results of other workers [6, 7]. It seems, therefore, that an increased extrarenal drug elimination (see below) compensates the decreased renal excretion. As a result of these two opposite processes, the kinetics of MTX appear to be linear in the tested dose range. Exhaustion of the extrarenal compensation can explain nonlinear kinetics in rabbits [7], other animal species [2, 24, 28], and man [17, 20, 21].

After IV administration MTX was eliminated by renal excretion in unchanged form (50%) and as 7-OH-MTX (15%). Excretion into bile and feces [11, 25] and formation of MTX polyglutamates stored intracellularly [26] may account for the missing 35% of the MTX dose.

In rabbits the renal MTX clearance (3–4.5 ml/kg × min) is similar to the inulin clearance (4.1 ml/kg × min; personal, unpublished results). In other species the renal clearance for MTX is well above the glomerular filtration rate [10, 12, 18], owing to tubular secretion [1, 3, 10]. Tubular secretion was also demonstrated for the rabbit [7]. A low clearance value, however, does not exclude secretion, as tubular reabsorption, which is known in dogs [10], may compensate secretion.

In our experiments (short-term and continuous infusion) 15%–18% of the injected MTX was regained as 7-OH-MTX from urine, which is comparable to the data in man [4, 27]. The metabolized fraction was almost constant with the different doses and after repeated administration to the same animals, giving no evidence for enzyme induction by increasing MTX concentrations [21] or repeated MTX infusions [15, 16].

From observations in man [15] we had expected a slow elimination of 7-OH-MTX, but a biexponential function with a terminal half-life of only 0.45 h was sufficient to describe the kinetics of the drug after IV application. The distribution volume of 7-OH-MTX (Table 1) was rather small, indicating only limited distribution to peripheral tissues. About 80% of the injected dose was recovered from the urine in 5 h, while the total excretion was calculated to be nearly 100%. Thus, 20% of the injected 7-OH-MTX must be eliminated extrarenally, a figure that can also be derived from the K_f value and the renal excretion of 7-OH-MTX in the continuous infusion experiments with MTX. Excretion into the feces via the bile is a possible explanation [22], but Chen and Chiou [5] have reported that biliary secretion of 7-OH-MTX is negligible in the rabbit. They regained nearly 100% of the administered dose from urine during a 20 h collection period [6] and concluded that there must be a slow terminal elimination phase that cannot be demonstrated in plasma because the 7-OH-MTX concentrations are below the detection limit of the HPLC method. We did not collect urine for long enough to confirm or to rule out this hypothesis. It is difficult to compare the results of Chen and Chiou (large volume of distribution; large total body clearance) to our data, because they are based on a different (three-compartment) kinetic model.

There are many complex models for the kinetics of MTX [2, 20, 28], but our results justify the attempt to calculate 7-OH-MTX concentrations after MTX injection with the simple assumption of linear kinetics for MTX (three compartments) and the metabolite (two compartments). In this way the 7-OH-MTX concentration in plasma is predicted reasonably well for the first 4 h after MTX infusion (Fig. 5). Later, the measured concentrations of 7-

OH-MTX exceed the concentration of MTX and are much higher than predicted. This discrepancy can be explained by several additional assumptions. First, there may be a third (certainly intracellular) compartment for 7-OH-MTX that is not seen after a single IV injection because there is no detectable γ-elimination phase [6]. In this case, a γ-phase would become apparent after MTX injection if a sufficient amount of 7-OH-MTX was formed in this intracellular space, possibly from stored MTX polyglutamates [8]. Alternatively, 7-OH-MTX may undergo an enterohepatic circulation in which case the small amount reabsorbed after an IV administration would again go undetected. After MTX injection, however, the liver could transform a considerable fraction of the dose (perhaps the 35% eliminated extrarenally) to 7-OH-MTX and excrete it directly into the bile. Reabsorption could then sustain an observable, long-lasting elimination phase. Both explanations are highly speculative. But it is obvious that a detailed knowledge of the hepatic and extrahepatic 7-OH-MTX formation and the enterohepatic circulation of 7-OH-MTX in rabbits could also lead to a better understanding of the 7-OH-MTX kinetics in man where the half-life of the metabolite is usually longer than that of the parent compound [4, 15].

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