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Renal and hepatic toxicity after high-dose 7-hydroxymethotrexate in the rat

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Abstract. To examine directly the hepatic and renal toxicity of 7-hydroxymethotrexate (7-OH-MTX) without interference of the parent compound methotrexate (MTX), we purified and gave 100 mg/kg 7-OH-MTX to rats, a dose resulting in serum levels of 7-OH-MTX comparable with those achieved in the clinic after the administration of high-dose MTX (HD-MTX). After only 5 h, the 7-OH-MTX-treated rats demonstrated 2.6-fold increases in serum creatinine values and 2-fold elevations in serum aspartate aminotransferase (ASAT) levels as compared with the controls. Morphologic evidence of toxicity, however, was apparent only in the kidneys. Intraluminal cellular debris containing membranous material and deteriorated organelles was seen, but no precipitate of the delivered drug. The peak serum concentration of 7-OH was up to 939 μM, and concentrations of 7-OH-MTX declined triphasically, showing a $t_{1/2\alpha}$ value of 2.45 min, a $t_{1/2\beta}$ value of 30.5 min, and a terminal half-life ($t_{1/2\gamma}$) of 240 min. The total clearance value was 14.5 ml min^{-1} kg, and the postdistributional volume of distribution (V_β) was 5070 ml/kg. Our results may indicate a direct toxic effect of 7-OH-MTX on kidney and liver cells.

Introduction

The antifolate methotrexate (MTX), used in high doses in several chemotherapy regimens [4, 15, 38], results in notable concentrations of 7-hydroxymethotrexate (7-OH-MTX) in serum and urine [5, 6, 14, 23, 27, 36]. The metabolite was initially considered a detoxification product [25, 26, 32], since it was found to be 200 times less cytotoxic than the parent compound [17]. However, MTX-induced nephro- and hepatotoxicity have been associated with this metabolite [10, 22, 23].

Acute hepatotoxicity is frequently observed in humans subjected to high-dose MTX (HD-MTX) therapy [3, 7, 19, 24, 41]. 7-OH-MTX has recently been suggested as a possible mediator of MTX-induced acute hepatotoxicity in the rat [10]. As the metabolite is 3–5 times less soluble than MTX in aqueous solution, intratubular crystallization of 7-OH-MTX is proposed as one mechanism of renal damage after MTX therapy [22, 23, 39].

Hitherto, the exact role and mechanism of 7-OH-MTX in renal and hepatic toxicity relative to that of MTX has remained obscure. To examine directly the hepatic and renal toxicity of 7-OH-MTX without interference of the parent compound MTX, we purified and gave 100 mg/kg 7-OH-MTX to rats, a dose resulting in serum levels of 7-OH-MTX comparable with those achieved in the clinic after the administration of HD-MTX [5, 6, 14].

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Materials and methods

Drugs and chemicals. 7-OH-MTX reference standard was a gift from Dr. F. M. Sirotnak (Memorial Sloan-Kettering Cancer Center, USA). MTX was a gift from Nycomed Pharma (Oslo, Norway). Methanol and tetrahydrofuran (both of high-performance liquid chromatography grade) were obtained from Rathburn Chemicals (Walkerburn, UK). Acetic acid was supplied by E. Merck (Darmstadt, Germany), and ammonium acetate was obtained from May & Baker Ltd. (Dagenham, UK). Water was distilled on a Milli-Q (Millipore) water purification system. All solutions containing 7-OH-MTX were stored under protection from light at –20° C.

Preparation of 7-OH-MTX. Urine was collected from one patient receiving HD-MTX chemotherapy (33.6 g/m^2). The urine was precipitated with concentrated acetic acid and kept on ice prior to centrifugation at 4000 *g* for 20 min at 4° C in a Sorvall RC-5 equipped with a GSA rotor. Pellets were resuspended in 1 *M* NaOH and centrifuged once more at 500 *g* for 10 min in an MSE centrifuge. The pH of the supernatant was adjusted to 6.0 with acetic acid. Methanol was then added to a final concentration of 10%, and the solution was filtered before its injection onto the high-pressure liquid chromatograph

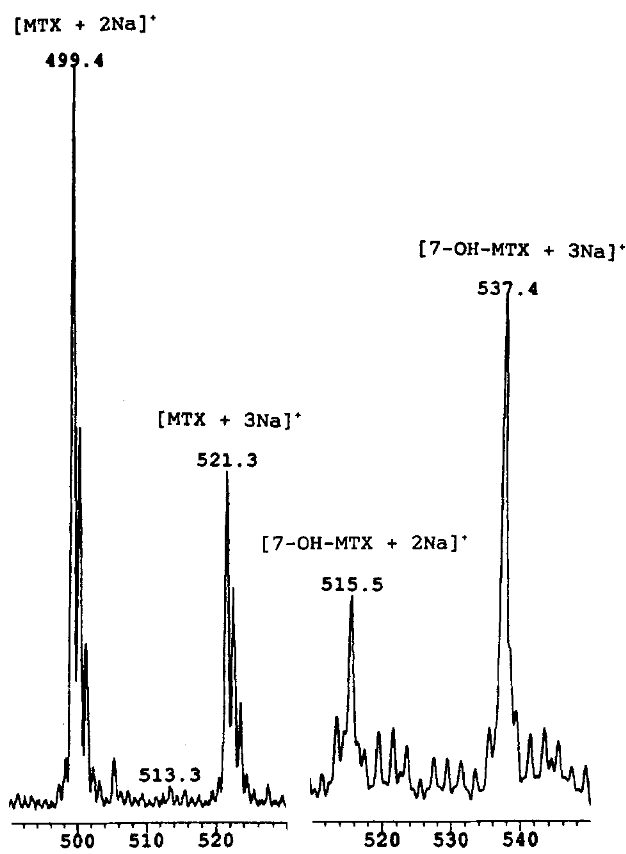

Fig. 1. FIB spectra of a reference compound of MTX (*left*) and our prepared 7-OH-MTX (*right*), showing Na adducts of MTX and 7-OH-MTX

(HPLC). Our HPLC system consisted of a Perkin Elmer Series 4 liquid chromatograph, a Rheodyne 7125 injector fitted with a 2-ml preparation loop, a 1- × 50-cm Techsil 10 C_{18} column, a Kratos Spectroflow 773 absorbance detector, and a Spectra-Physics SP 4270 integrator connected to a Pharmacia Frac-200 fraction collector.

The solvent system comprised 0.01 *M* ammonium acetate (pH 6.0), 9% methanol, and 2% tetrahydrofuran. The flow rate was 5.0 ml/min and detection was carried out at 306 nm. An adequate separation between 7-OH-MTX and MTX was obtained with a retention time of 24 min for 7-OH-MTX. Fractions containing 7-OH-MTX were pooled and lyophilized, washed twice with ice-cold water and once with ice-cold acetone, and dried over phosphorus pentoxide. The purity of 7-OH-MTX was higher than 94% as measured by HPLC. Mass spectra were obtained by fast ion bombardment (FIB) (Fig. 1) performed on a VG Tribrid using samples dissolved in 10% 0.1 *N* NaOH in glycerol.

Animals and operation. Nonfasted male Wistar rats weighing 190–210 g (Charles River, WIGA GmbH, Sulzseld, Germany) were used for the experiments. Ten rats were allocated to two groups: five rats were subjected to 7-OH-MTX infusion, whereas the other five served as controls. The rats were anesthetized with 0.4 mg/kg fentanyl given i.p. (maintenance dose 0.1 mg kg^{-1} h^{-1} given i.m.) and had their right external jugular vein cannulated [8].

Experiment. 7-OH-MTX was suspended in NaOH and HCl was added to a final concentration of 10 mg/ml (pH 8.9). Drug and diluent (at identical pH) were given as short-term infusions of 10 min through the venous catheter. One rat receiving 7-OH-MTX died 100 min postinfusion.

Blood samples (200 µl) for 7-OH-MTX analysis were drawn at the times indicated in the figure showing serum concentrations of 7-OH-MTX (see Fig. 5). The catheters were flushed with heparinized saline (10 IU/ml) immediately after infusions and after each blood collection. Blood for enzyme and creatinine analyses were obtained after cannulation of the jugular vein and at 5, 8, and 10 h postinfusion. Voided urine was collected, and after the animals had been killed, the urinary bladder was aspirated to ensure complete collection. The pH was measured in all urine samples.

During the experiment the rats were hydrated with 0.06 *M* $NaHCO_3$ in isotonic saline delivered at 6 ml kg^{-1} h^{-1}. Venous blood-gas analyses were performed before the start of the infusion and at the end of the experiments. Hematocrit analyses have been performed in our earlier studies using the same model [8–10], showing a reduction of about 20% in both treated and control rats during the experiment. At 10 h, the animals were killed and subjected to laparotomy and exsanguination. The left kidney and one specified liver lobe were immediately removed for morphologic examination. Tissue samples (0.2–1.5 g) from three of the 7-OH-MTX-treated rats were obtained from the kidney, liver, brain, heart, lung, testis, and gluteal muscle and were washed in ice-cold saline, weighed, and homogenized with a Potter-Elvehjem homogenizer.

Analytical procedures. Analysis of the 7-OH-MTX concentration in serum and urine was performed by reverse-phase HPLC as reported previously [8, 10]. Because of interference, a modification of an ion-exchange HPLC method [11] was used for analysis of the tissue samples.

Serum aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) levels were determined using a BM/Hitachi 737 apparatus at 37° C [12]. The same instrumentation was used to determine serum creatinine concentrations with a kinetic Jaffe method. The reagent kits were supplied by the manufacturer (Boehringer Mannheim, Germany).

For light microscopy, the rat tissue was immersion-fixed in 4% phosphate-buffered formaldehyde, dehydrated, embedded, cut at 5 µm, and stained with hematoxylin and eosin and van Gieson's solution. Since this fixation liquid may dissolve precipitated 7-OH-MTX [30, 39], tissue fixation was also performed with an alcohol-based fixative. McDowell's fixative and 1% aqueous OsO_4 were used to prepare tissue for electron microscopy. The tissue was block-stained in uranyl acetate, dehydrated in graded ethanol, and embedded in Epon/Araldite. Ultrathin sections were cut on a Reichert ultracut, the contrasted in 5% aqueous uranyl acetate and Reynold's lead citrate prior to examination in a Jeol 1200 EX electron microscope.

Calculations. Serum concentrations of 7-OH-MTX were analyzed according to a three-compartment open model. The pharmacokinetic parameters were obtained by means of linear regression analysis in a semilogarithmic data set as reported previously [9]. Statistical analyses were performed using Student's *t*-test. Statistical significance was defined as $P < 0.05$. All results are expressed as mean values ± SD.

Results

Serum creatinine, ASAT, and ALAT

Serum levels of creatinine, ASAT, and ALAT are shown in Fig. 2. At 5 h postinfusion the 7-OH-MTX group demonstrated serum creatinine levels 2.6 times higher than the control values. Serum creatinine levels were significantly different at all time points investigated following the infusion, with serum concentrations being 59 ± 15.9 µmol/l in 7-OH-MTX-treated rats and 25 ± 4.7 µmol/l in controls at the end of the experiments. Further evidence of renal impairment was microscopic hematuria occurring in all rats receiving 7-OH-MTX (data not shown).

Serum ASAT concentrations were significantly elevated at 8 and 10 h after 7-OH-MTX infusion as compared with

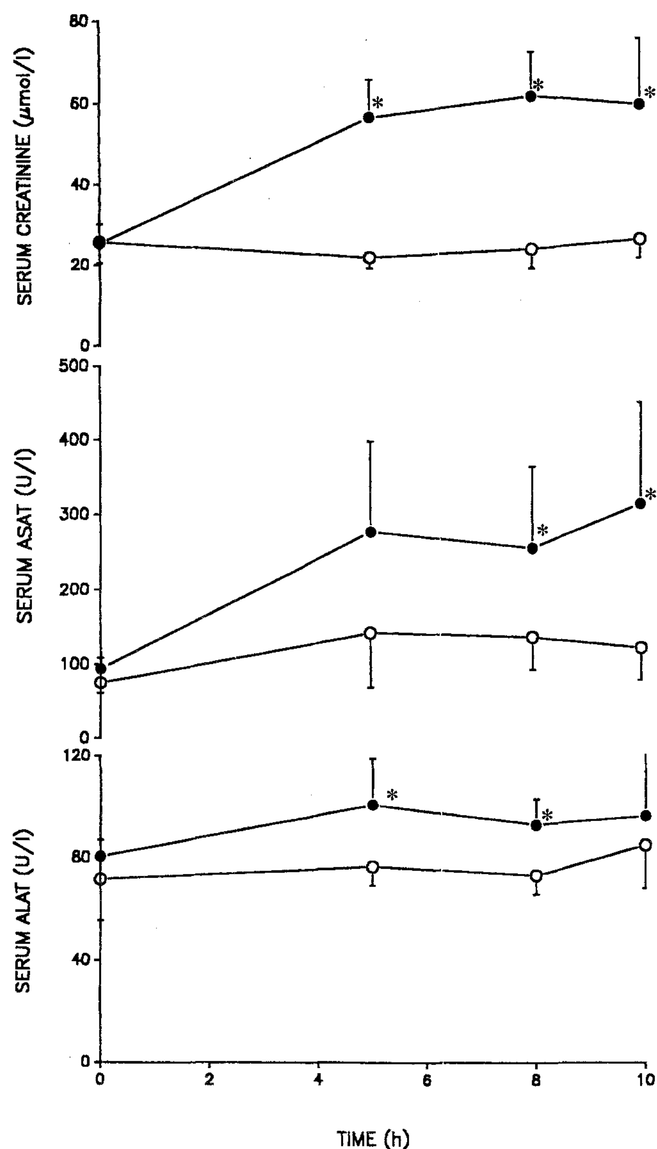

Fig. 2. Serum concentrations of creatinine (*top panel*), aspartate aminotransferase (*ASAT, middle panel*) and alanine aminotransferase (*ALAT, bottom panel*) versus time as determined prior to and after short-term infusions of 100 mg/kg 7-OH-MTX (*filled symbols*) or diluent (*open symbols*) in anesthetized rats. Data represent mean values ± SD (n = 4). $^{*}P < 0.05$

controls, with peak levels (10 h) being 313±136 and 121±43 U/l, respectively. Serum ALAT levels were slightly elevated in 7-OH-MTX-treated rats, with significantly higher values being recorded at 5 and 8 h post-infusion.

Morphologic changes detected after 10 h

Examination by light microscopy (LM) revealed slight to moderate changes in the kidneys of rats that had received 7-OH-MTX. The epithelium of the proximal tubules was swollen and some vacuoles were present. Blurring of the epithelial lining and cytoplasmic protrusion into the lumina were observed. Cellular casts were demonstrated, particularly in the distal tubules in scattered areas (Fig. 3). No obvious change was observed in the glomeruli.

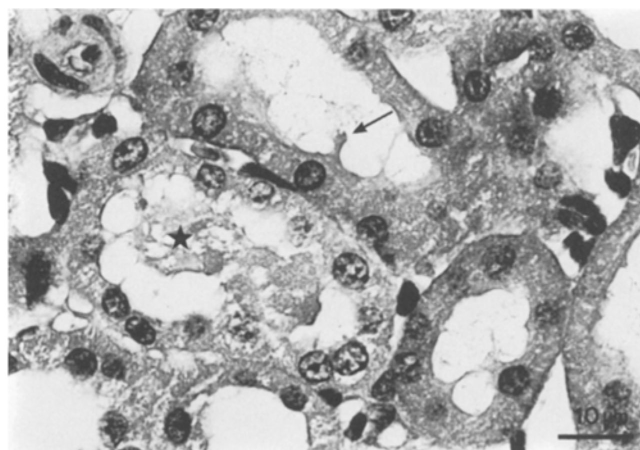

Fig. 3. Light microscopic picture of kidney tubuli of a rat obtained 10 h after 7-OH-MTX administration. A distal tubulus containing cellular debris (*star*) and another tubulus with cytoplasmic protrusion into the lumen (*arrow*) can be seen

Table 1. Pharmacokinetic variables determined in rats given 10-min i.v. infusions of 100 mg/kg 7-OH-MTX

Variable	Value
$t_{1/2\alpha}$ (min)	2.45 ± 0.66
$t_{1/2\beta}$ (min)	30.5 ± 5.6
$t_{1/2\gamma}$ (min)	240 ± 18
V_c (ml/kg)	366 ± 64
V_β (ml/kg)	5070 ± 1479
Cl_T (ml min^{-1} kg)	14.5 ± 3.2

Data are expressed as mean values ± SD (n = 4)

Table 2. Tissue/serum concentration ratios determined at 10 h in 3 rats given 100 mg/kg 7-OH-MTX

Tissue	Tissue/serum ratio (n = 3)	
Kidney	2799	(639 –6011)
Liver	23.1	(3.2 – 38.3)
Brain	0.06	(0.05– 0.08)
Testis	0.53	(0.45– 0.6)
Lung	0.85	(0.44– 1.66)
Heart	0.22	(0.07– 0.37)
Muscle	0.43	(0.22– 0.77)

Data are given as mean values; ranges are shown in parentheses

Electron microscopy (EM) further outlined the changes visible by LM. Tubular ductuli showed lumina with cellular debris containing membranous material and deteriorated organelles (Fig. 4A). Racket-like structures protruded from the epithelial cell surface into the lumina in many tubules (Fig. 4B). There were several slit-like spaces between the epithelial cells in addition to apical vacuoles (Fig. 4C). The microvilli appeared to be unaffected and the mitochondria were only slightly enlarged. Examination of the liver by LM or EM did not demonstrate changes in rats treated with 7-OH-MTX.

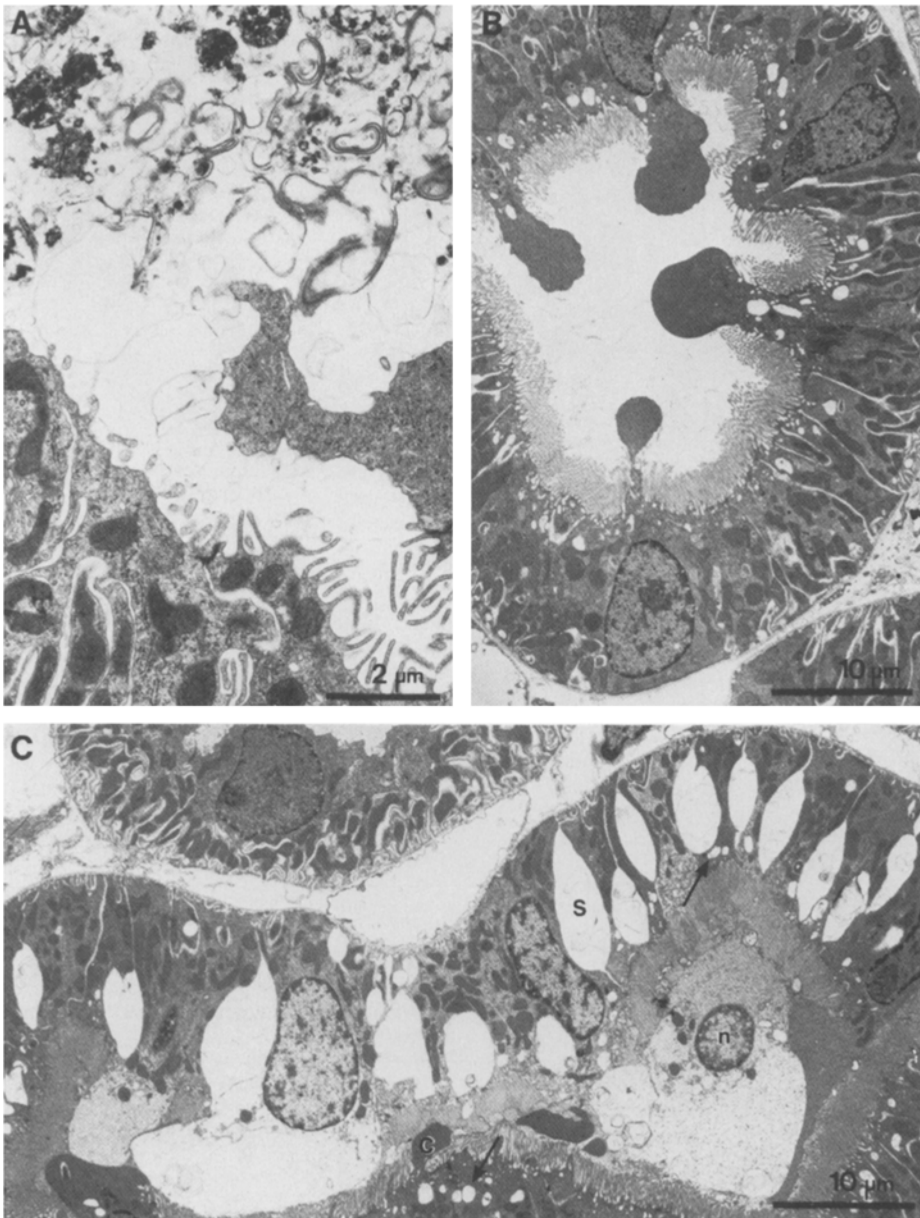

Fig. 4 A–C. Electron microscopic details observed in specimens obtained from rats 10 h after the administration of 100 mg/kg 7-OH-MTX. **A** Electron micrograph of a part of a distal tubulus. Cellular debris containing membranous material and deteriorated organelles are present in the lumen. **B** Transverse section of a proximal tubulus, showing several racket-like cytoplasmic structures protruding from the epithelial cells into the lumen. **C** Transverse section of a proximal tubulus. Numerous small, apically located intracytoplasmic vesicles (*arrows*) in addition to slit-like spaces between epithelial cells (*S*) can be seen. Cytoplasmic protrusions (*c*) as well as cellular debris and an empty nuclei (*n*) are visible within the lumen

Pharmacokinetics and distribution of 7-OH-MTX

Serum concentrations of 7-OH-MTX reached 939 µ*M* and declined triphasically (Fig. 5); the pharmacokinetic variables are detailed in Table 1. Drug concentration ratios between tissue and serum as determined at the termination of the experiment are presented in Table 2. The kidney/serum ratio was substantial at 2799 ± 2836 (range, 639–6011 µ*M*), whereas the brain/serum ratio was only 0.06 ± 0.01. Blood-gas values were comparable in drug-treated and control rats at the beginning and the end of the experiments.

Renal excretion of 7-OH-MTX

The urinary recovery of 7-OH-MTX during the experiments (10 h) was $3.01\% \pm 0.96\%$ of the delivered dose. A slight but not statistically significant difference in the voided volume of urine was observed between the two groups (mean, 9.1 ml in controls vs 9.9 ml in rats treated with 7-OH-MTX). The urinary pH increased slowly in both groups due to the $NaHCO_3$ given for hydration, reaching mean pH values of 6.49 in controls and 6.95 in 7-OH-MTX-treated rats.

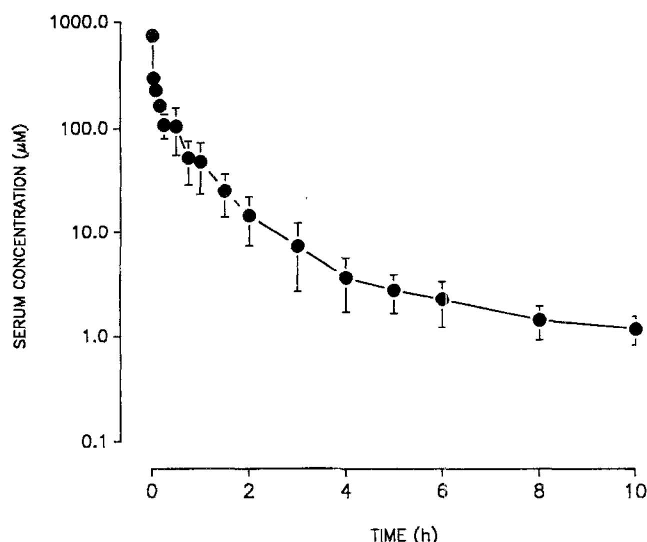

Fig. 5. Serum concentrations of 7-OH-MTX versus time as determined following short-term infusions of 100 mg/kg 7-OH-MTX in anesthetized rats. Data represent mean values ± SD (n = 4)

Discussion

This is the first report of acute nephrotoxicity in an animal model after 7-OH-MTX administration at doses relevant for HD-MTX treatment in humans. The observation that the serum creatinine levels in rats given 7-OH-MTX exceeded the control values by a factor of 2.6 as early as 5 h post-infusion as well as the microscopic hematuria and morphologic changes detected within 10 h in the kidney indicate severe renal damage.

Renal injury remains a highly feared side effect of HD-MTX treatment [18, 31, 40]. The underlying mechanism remains a matter of controversy [1, 2, 13, 20, 34, 35]. Several mechanisms have been postulated, of which tubular precipitation producing obstructive renal failure is the most widely accepted [1, 29, 35, 39]. Our results showing no tubular precipitation of 7-OH-MTX, in contrast to those of Jacobs et al. [22, 23], could be suggestive of a direct toxic effect of 7-OH-MTX on the tubular system of the kidney. Further studies will have to focus on delineating the topographic and functional aspects of 7-OH-MTX-induced kidney damage.

We have previously reported acute hepatotoxicity after HD-MTX treatment in bile-cannulated rats [10] and proposed 7-OH-MTX as a notable contributor. Acute hepatotoxicity, which has frequently been reported after HD-MTX therapy [33, 42, 43], was also observed in the present investigation. Biliary precipitation of 7-OH-MTX, however, was not detected. This may have been due to the rapid decline in serum 7-OH-MTX concentration noted after the infusion as compared with the prolonged high 7-OH-MTX levels observed in rats given HD-MTX [10]. In our former study [10], transaminase levels measured in rats treated with 1000 mg/kg MTX were comparable with those found in controls. The observation that ASAT (and ALAT) levels were significantly elevated in rats treated with 100 mg/kg 7-OH-MTX may indicate that the metabolite is a more potent hepatotoxic compound than the parent drug. In accordance with the previous report of MTX-associated acute hepatotoxicity in humans [28], we found serum ASAT values to be the most sensitive variable for acute liver injury in rats given 7-OH-MTX. The transaminase elevations however, were not accompanied by morphologic changes in the liver pointing to a limited acute hepatotoxicity.

There are only two reports of the pharmacokinetics of 7-OH-MTX after its administration in the rat, albeit at lower doses than those employed herein. Slørdal et al. [37] and Fahrig et al. [16] described 7-OH-MTX pharmacokinetics after doses of 1 and 4 mg/kg, respectively. Following infusions of 100 mg/kg 7-OH-MTX, the drug was eliminated in a triphasic manner, whereas in the above-mentioned studies [16, 37] the pharmacokinetics were calculated according to a two-compartment model. A significantly greater postdistributional volume of distribution (V_β) was calculated after our high dose as compared with those used in the previous studies [16, 37]. Slørdal et al. [37] found equal V_β levels for MTX and 7-OH-MTX in the rat, contrary to their estimations in man [36]. Fahrig et al. [16] also found similar values for 7-OH-MTX and MTX, whereas Iven et al. [21] demonstrated a 7-fold lower V_β value for 7-OH-MTX as compared with MTX in the rabbit. In contrast, we found the V_β value (Table 2) to be somewhat higher for 7-OH-MTX as compared with MTX [10], which corroborates the greater tissue/serum ratios obtained in the kidney and liver (Table 2). The high concentration of 7-OH-MTX detected in renal tissue devoid of precipitation may support the hypothesis of a direct toxic effect of 7-OH-MTX [1, 35].

The urinary recovery of 7-OH-MTX was low, with only 3.01% of the delivered dose being excreted in the urine during the 10-h experiment. Slørdal et al. [37] found 22% of a 1-mg/kg dose of 7-OH-MTX in the urine after 2 h, whereas Fahrig et al. [16] recovered 11% of a 4-mg/kg dose in the urine over a 200-min period. This inverse relationship between the 7-OH-MTX dose and the urinary recovery of the drug may be indicative of a dose-dependent saturation of the renal excretion of 7-OH-MTX or, possibly, also of a reduction in 7-OH-MTX excretion secondary to renal damage occurring after the high dose of 7-OH-MTX. It should be borne in mind, however, that the urine-sampling period was too short to reflect the total cumulative urinary excretion of 7-OH-MTX.

Measurements of 7-OH-MTX in bile were not performed in this study, but the low urinary output, the low $t_{1/2}$ values obtained for the α- and β-phases, and the previous report of biliary recovery amounting to as much as 73% of the delivered dose [16] suggest that considerable amounts of 7-OH-MTX undergo biliary excretion in the rat.

We obtained further evidence that 7-OH-MTX is a major contributor to the nephro- and hepatotoxicity associated with MTX treatment. Our results may also indicate a direct toxic effect on kidney and liver cells in contrast to the most widely accepted theory of precipitation as a major mechanism of toxicity [10, 29, 35, 39].

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