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Evaluation of methotrexate tissue exposure by in situ microdialysis in a rat model

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Abstract. The feasibility of using a microdialysis technique to obtain pharmacokinetic data on tissue exposure to methotrexate (MTX) was investigated. Microdialysis probes were implanted in the jugular vein, femoral muscle, and liver of anesthetized male Wistar rats. MTX (100 mg/kg) was given as a bolus injection through an indwelling venous catheter, and blood samples were obtained through a second venous access and by microdialysis for a total of 6 h. Heparinized plasma, ultrafiltered plasma, and microdialysis effluent from tissue and venous probes were analyzed by high-performance liquid chromatography. Centrifugal ultrafiltration of rat plasma spiked in vitro with MTX (1–100 μ*M*) revealed a mean binding to plasma proteins of 21%. In vitro microdialysis of this spiked plasma resulted in 23% relative recovery of the unbound fraction. In rats receiving MTX, plasma protein binding was 23% and the relative drug recovery as assessed with venous microdialysis probes was 18%. Plotting of unbound (i.e., ultrafiltrate) MTX concentrations in the blood against venous microdialysis perfusate values in the blood gave a good linear correlation with a coefficient of correlation (r^2) of 0.98. There was also a linear correlation between the total MTX concentrations in venous blood and the drug levels in microdialysis samples from muscle and liver (r^2 = 0.93 and 0.74, respectively). Area under the curve estimations were consistent with an MTX exposure of 30% and 46% for the muscle and liver as compared with the circulation. The present study demonstrates that the microdialysis technique can provide reproducible data on tissue exposure to MTX in an animal model and indicates that the methodology is adaptable to clinical settings.

Key words: Microdialysis – Methotrexate – Tissue – Rat

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Introduction

Therapeutic drug monitoring relies almost entirely on the determination of drug levels in plasma or serum. This practice rests on the assumption that a stable equilibrium exists between the concentration of drug in the bloodstream and that at the site of action. Drug therapy in oncology is crucially dependent on the maintenance of adequate dose intensity in target tissues. When local factors peculiar to tumor tissue are taken into account, a steady state for the in vivo penetration of anticancer agents into solid tumors may at best be subject to considerable interindividual variation.

Techniques for the direct monitoring of drug concentrations in tissue have not been generally available, and most studies have relied on the determination of drug levels in tissue obtained through biopsy or surgery. Although this approach offers the advantage of monitoring intracellular drug concentrations, it rarely allows serial sampling. This may partially explain the paucity of data on drug pharmacokinetics in solid tissues.

The recent development of in situ microdialysis has made possible the on-line monitoring of xenobiotics and endogenous substances in extracellular spaces, including tumor tissue. The technique is based on the principle of passive transport of compounds across a dialysis membrane. The microdialysis probe, a double tube with a semipermeable membrane at one end, can be implanted in virtually any compartment without causing extensive tissue damage. A pump regulates the flow of a perfusate that removes molecules diffusing across the membrane from the biophase, and subsequent analysis of the perfusate can permit an assessment of the extracellular levels of the particular compound involved. Membranes with different relative molecular-weight cutoffs are commercially available. The method has thus far been employed for measuring endogenous substances in muscle and brain tissue in humans but to our knowledge has not been implemented for the in vivo quantitation of drugs [1].

The antifolate methotrexate (MTX) is one of the most widely used cytotoxic drugs. High doses of this compound have been successfully employed in the treatment of leu-

kemias and various solid tumors, and its pharmacokinetics has been extensively studied [6]. We investigated the feasibility of obtaining pharmacokinetic data on tissue exposure to MTX by employing the microdialysis technique in normal rats given high doses of the drug by the intravenous route.

Materials and methods

Drugs and chemicals. Unformulated MTX was obtained from Lederle Laboratories (Pearl River, N. Y., USA). 7-Hydroxymethotrexate (7-OH-MTX) was a kind gift from Dr. F. M. Sirotnak (Memorial Sloan-Kettering Cancer Center, New York, N. Y., USA). Flexible microdialysis probes (10-mm membranes with a diameter of 0.50 mm; 20-kDa cutoff size) were obtained from CMA/Microdialysis (Stockholm, Sweden). The perfusion solution (Ringer acetate; Kabi Pharmacia AS, Halden, Norway) was delivered at ambient temperature by a CMA/100 syringe pump, and the perfusate was collected by a CMA/140 fraction collector. High-performance liquid chromatographic (HPLC)-grade methanol was obtained from Rathburn Chemicals Ltd. (Walkerburn, UK). All other reagents were of analytical grade.

In vitro experiments. To determine drug recoveries in vitro, freshly obtained heparinized rat plasma was spiked with MTX and subjected to microdialysis at 37° C with gentle stirring. Estimations were made at three different MTX concentrations: 1, 10, and 100 μ*M*. In some experiments, 7-OH-MTX was added to the plasma samples at concentrations of one-tenth of the MTX levels. Plasma ultrafiltrates were obtained before and after dialysis by centrifugation of samples at 3,600 *g* for 30 min through a 30-kDa cutoff ultrafilter (Ultrafree MC. Millipore, Bedford, Mass., USA) in a Microfuge 13 (Heraeus Sepatec, Osterode, Germany). Albumin was measured before and after microdialysis by the bromcresol green colorimetric method (Kodak Ektachem 700XR, Eastman Kodak Co., Rochester, N. Y., USA).

In vivo experiments. Male Wistar rats weighing 270–325 g were obtained from Shaw's Farm (Oxon, UK) and maintained in conventional facilities. Animals were anesthetized by subcutaneous injection of 0.125 ml of a mixture of fentanyl (0.2 mg/ml) and fluanisone (10 mg/ml) and 0.125 ml midazolam (5 mg/ml). A polyethylene catheter (PE-10; Clay Adams, Becton & Dickinson, Rutherford, N. J., USA) inserted into the left jugular vein was used for MTX administration and, after coupling to a peristaltic pump (IPN-12; Ismatec SA, Glattbrugg-Zürich, Switzerland) for delivery of maintenance anesthesia (1.3 μl/min of the mixture used for induction).

A second catheter (diameter 1.0 mm; Viggo-Spectramed, Helsingborg, Sweden) was inserted into the left femoral vein and used for blood sampling. Three microdialysis probes were inserted in the right jugular vein, the adductor muscle of the right femur, and the right liver lobe, respectively. Incisions were closed with Novafil 3-0 (Davis+Geck, Hampshire, UK) sutures. The surgical procedures took about 1 h. Subsequently, the animal was put onto a CMA/150 temperature controller set at 37.5° C. Before the start of the experiments the microdialysis probes were flushed with Ringer acetate at 20 μl/min to purge the membranes and tubing of air bubbles. At 30 min before drug administration the perfusate flow was reduced to 4 μl/min. MTX was given as a 1.0-ml bolus injection of 2-min duration.

Samples and sample treatment. Samples of 120-μl dialysis fractions were obtained continuously for 6 h. Blood samples (300 μl) were drawn in the middle of dialysis sample periods at 15, 45 60, 75, 105, 165, 225, 285, 345, and 360 min and replaced with an equal amount of Ringer acetate. The blood was transferred to 1.5-ml microcentrifuge tubes containing 14 μl heparin (25,000 IU/ml; Nycomed, Oslo, Norway) and was centrifuged at 5,600 *g* for 5 min. At different time points (15–343 min), between 1 and 3 (total, 14) plasma samples from the 6 animals were additionally subjected to ultrafiltration centrifugation as outlined above. Plasma and ultrafiltrates were stored frozen and protected from light until analyzed. Sample preparation prior to chromatography consisted of deproteinization by the addition of a one-fifth volume of 2 *M* perchloric acid, vigorous mixing, centrifugation at 15,000 *g* for 5 min, and transfer of samples to borosilicate glass autosampler vials (Chromacol Ltd., London, UK).

Table 1. Protein binding and microdialysis recovery of MTX in rat plasma in vitro and during in vivo experiments

	In vitro	In vivo
Bound MTX (%)	21.1 ± 7.8	22.8 ± 10.9
Relative recovery of MTX (%)	22.7 ± 3.1	17.9 ± 3.9

Data are given as mean values ± SD (n=6)

Analytical procedure. MTX and 7-OH-MTX levels were quantified by a modified isocratic HPLC assay described in detail elsewhere [11]. The chromatography equipment was produced by Shimadzu Corporation (Tokyo, Japan). The solvent-delivery system consisted of a DGU-3A on-line degasser coupled to a LC-9A quaternary gradient pump. The column temperature was maintained using a CTO-6A column oven and on on-line solvent preheater. Samples were injected by an SIL-9A autoinjector maintained at ambient temperature and detection was carried out by an SPD-6AV variable-wavelength UV detector. Peak area integrations were performed by a Chromatopac C-R6A integrator.

Chromatography was performed on a Supelcosil C18 octadecyldimethylsilyl (ODS) column (4.6 × 150 mm; particle size, 3 μm; Supelco, Bellefonte, Pa., USA) equipped with a 20-mm Supelguard. The mobile phase consisted of a TRIS-sodium dihydrogen phosphate (both 0.1 *M*, pH 6.7): methanol mixture (80:20, v/v). The mobile phase was delivered at a rate of 1 ml/min and the column temperature was maintained at 40° C. The UV detector was operated at 370 nm. Either 50 or 100 μl of sample was injected. Between analyses the autoinjector line was washed by flushing with a 60% aqueous methanol solution.

Calculations. The area under the time-concentration curve (AUC) was calculated by the trapezoidal rule. The calculation rests on the assumption that $AUC_{15-\infty} >> AUC_{0-15}$, hence making the 0- to 15-min area negligible. Total clearance was calculated by the equation $Cl_{tot} = Dose/AUC$. Half-lives were calculated by the equation $t_{1/2} = \ln 2/k_e$, where k_e is the elimination rate constant. Apparent volumes of distribution were obtained by the equation $V_D = Cl_{tot}/A+B$, where *A* and *B* are the zero-time intercepts of the extrapolated regression lines of the α and β phases, respectively. Statistical analyses were performed by least-squares regression.

Results

The protein binding and relative recovery of MTX in vitro and in vivo are given in Table 1. In vitro experiments were undertaken using heparinized rat plasma at 37° C with gentle stirring.

The degree of plasma protein binding was assessed by HPLC measurement of total and ultrafiltered plasma, whereas recovery was determined by comparison of the amount of drug in the microdialyzed samples with the concentrations of unbound drug in the plasma (i.e., ultrafiltrate). Albumin levels did not change significantly during in vitro microdialysis (data not shown).

Preliminary in vitro experiments were undertaken to determine the kinetics of drug diffusion across the microdialysis membrane. Equilibrium conditions were reached

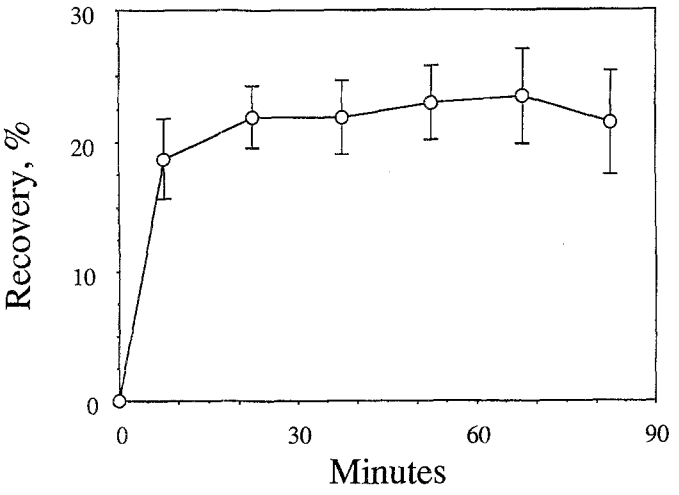

Fig. 1. Time-dependent equilibration during microdialysis of rat plasma in vitro. MTX (1, 10, and 100 μ*M*) was added to plasma, and microdialysis was undertaken at 37° C with gentle agitation. Data are expressed as means values ± SD (n = 3)

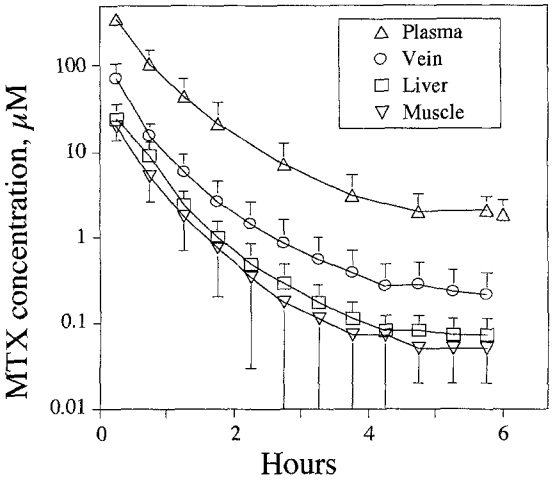

Fig. 2. MTX concentration in plasma and microdialysis perfusates from venous blood, muscle, and liver after bolus injection of 100 mg/kg of the drug in rats. Microdialysis data were not corrected for recovery. Data are expressed as mean values ± SD (n = 6)

within 30 min (Fig. 1) and were independent of the absolute MTX concentrations in the plasma. Similar results were observed after microdialysis of 7-OH-MTX (data not shown).

The total plasma and tissue pharmacokinetics of MTX as determined after the administration of 100 mg/kg to rats are shown in Fig. 2. Data from microdialysis sampling of venous blood, liver, and muscle have not been corrected for recovery and represent relative values as opposed to the plasma drug levels. The pharmacokinetic profiles are in accordance with a two-compartmental model with a final elimination phase from 2 h and onward. The exposure to unbound MTX in the different compartments was calculated by comparing the AUCs delineated by the plasma microdialysis values to those obtained from probes situated in the muscle and liver. The relative exposures of muscle

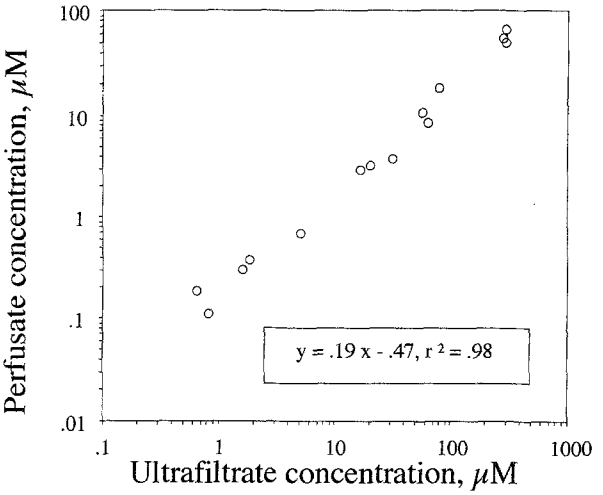

Fig. 3. Relationship between plasma ultrafiltrate (i.e., free fraction of the drug) and microdialysis perfusate concentrations of MTX in blood from rats. The line was constructed from pooled data points obtained at different time points after treatment of 6 animals with 100 mg/kg MTX. Microdialysis data were not corrected for recovery. Regression coordinates and correlation coefficient are given. Note the log scale

Table 2. Pharmacokinetic variables in rats given intravenous bolus injections of 100 mg/kg MTX

$t_{1/2\alpha}$ (min)	$T_{1/2\beta}$ (min)	V_D (l/kg)	Cl_{tot} (ml min^{-1} kg)	AUC (m*M* min)
30.0 ± 11.4	98.3 ± 39.3	3.2 ± 1.2	20.0 ± 6.0	11.9 ± 3.7

Data are presented as mean values ± SD (n = 6)

and liver tissues as compared with plasma were 30% and 46% (mean values, n = 6), respectively (Fig. 2). The pharmacokinetic parameters calculated from the total drug concentrations in plasma are given in Table 2.

The levels of unbound MTX as determined by centrifugal ultrafiltration of plasma samples were compared with the drug levels in microdialysis perfusates obtained from the jugular vein. The results are shown in Fig. 3. Linear regression analysis demonstrated a high degree of correlation (r^2 = 0.98) between ultrafiltered plasma and samples obtained by microdialysis.

The total MTX concentrations in plasma were compared with the levels measured in microdialysis effluents from both venous blood and femoral muscle and liver tissue. Figure 4 shows a highly linear relationship between the total plasma levels and the concentrations in microdialysis perfusate, with correlation coefficients (r^2) between the total plasma values and the dialysate values from blood, muscle, and liver being 0.82, 0.93, and 0.74, respectively.

The formation of the metabolite 7-OH-MTX varied considerably in individual rats. Figure 5 depicts MTX and 7-OH-MTX pharmacokinetics in a single animal. Total plasma concentrations of MTX were approximately 100 times higher than the corresponding 7-OH-MTX levels

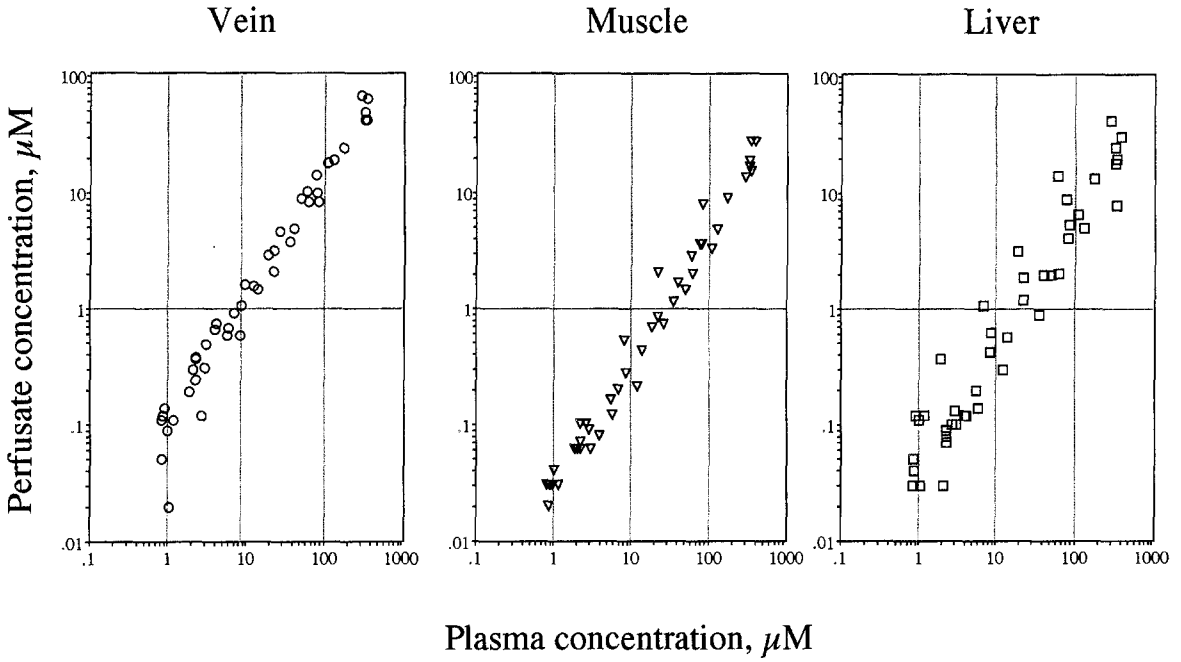

Fig. 4. Relationship between total plasma MTX levels and microdialysis perfusate concentrations obtained at different time points from 6 rats given 100 mg/kg of the drug. Microdialysis data were not corrected for recovery. Note the log scales. The correlation coefficients (r^2) between total plasma MTX levels and those in microdialysis fluid from blood, muscle, and liver are 0.82, 0.93, and 0.74, respectively

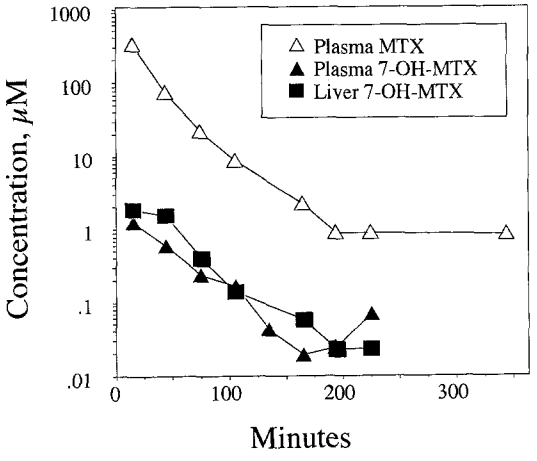

Fig. 5. MTX and 7-OH-MTX pharmacokinetics in one rat after treatment with 100 mg/kg MTX. 7-OH-MTX values obtained in microdialysis samples from liver were not corrected for recovery

at all time points. The levels of the 7-hydroxylated metabolite measured in the microdialysis effluent from liver (not corrected for recovery) were very similar to the total metabolite levels in the blood (Fig. 5).

Discussion

With a few notable exceptions, drug monitoring during anticancer chemotherapy has gained limited use. In light of the pronounced toxicity and narrow therapeutic index of most cytotoxic agents, the lack of analytically guided drug therapy is surprising. Its implementation however, has been hampered by a number of unresolved questions [7]. In particular, considerable problems are associated with the use of blood pharmacokinetic data to predict the treatment response in the individual patient.

Techniques to evaluate drug delivery to solid tumors could be of help in elucidating the complex relationship between the pharmacokinetic properties of and the response to cytotoxic agents. In this regard, microdialysis can provide direct access to extracellular spaces for monitoring tissue penetration of drugs. A priori to its clinical use is the demonstration that microdialysis can give reproducible pharmacokinetic data. Herein we report the results of microdialysis monitoring of MTX in three tissue compartments in normal rats.

Ultrafiltration studies in plasma spiked with MTX in vitro and obtained from rats after MTX treatment demonstrated a degree of protein binding of MTX of 21% and 23%, respectively. Microdialysis experiments showed a recovery of unbound MTX of 23% in vitro and 18% in the intact animals (Table 1). The absolute protein binding was constant over the range of MTX concentrations tested. The high degree of similarity between the in vitro and in vivo data shows that microdialysis can be employed for monitoring the unbound and presumably active drug during pharmacotherapy. The in vitro data correspond to what others have reported for therapeutic agents belonging to different classes [4, 5].

The relative recovery is the concentration of the substance in the perfusate when it leaves the probe, expressed as a percentage of the concentration in the surrounding medium [5]. Microdialysis of rat plasma in vitro demonstrated that a stable equilibrium was attained after 30 min (Fig. 1). The drug recovery in vitro was constant at different concentrations and comparable with the in vivo recovery (Table 1). Samples obtained from microdialysis

probes implanted in the circulation should thus reflect the blood concentration.

MTX was eliminated from the bloodstream of the rat in a biphasic manner (Fig. 2), with an initial phase with a mean half-life of 30 min being followed by a slower second phase with a half-life of 98 min (Table 2). We did not attempt to obtain blood samples to identify an initial distribution phase, reported to be of 5–10 min duration after intravenous treatment with MTX in the rat [2, 3]. Both phases thus represent elimination phases, and the samples were obtained during an apparent distribution equilibrium. With this in mind, the pharmacokinetic variables obtained after short-term MTX infusions in the rat (Table 2) are comparable with previously reported data [2, 3, 12]. The MTX AUCs expressing the exposure of liver and muscle tissue to the drug were compared with the AUCs of the venous perfusate values on the assumption that absolute drug recoveries are not highly dissimilar in the different compartments examined. The results may imply a lower degree of exposure to MTX for both liver and muscle tissue than for the blood cells.

A comparison of the MTX levels obtained in centrifuged ultrafiltrates of plasma and in microdialysis samples was undertaken to examine microdialysis recovery in vivo (Fig. 3). The high degree of correlation ($r^2 = 0.98$) between the unbound MTX in plasma and the drug concentrations in microdialysates shows that microdialysis techniques may be employed for measurement of drug levels in the circulatory compartment.

A comparison was made between the total plasma MTX concentrations and the levels measured in microdialysis probes in blood, liver, and muscle during apparent distribution equilibrium. The data implied that tissue concentrations closely followed plasma concentrations (Fig. 4). This was further substantiated by regression analysis, which revealed strong positive correlations between plasma and microdialysis data from venous blood, muscle, and liver. However, we present no experimental data to assess absolute drug recoveries in the muscle and liver compartments and cannot assume that these values are identical to the those observed in blood under in vitro or in vivo conditions. Indeed, one may speculate that diffusion rates in solid tissues such as muscle and liver are lower than those in liquid, and estimations of absolute tissue recoveries will need further investigation under steady-state conditions [9, 13].

In an animal that produced significant levels of the extracellular metabolite 7-OH-MTX the liver perfusate concentration of the metabolite (not corrected for recovery) was equal to or exceeded the plasma concentration (Fig. 5). The assumption of an absolute recovery of the metabolite of ≤20%, which seems reasonable on the basis of blood-recovery data for the parent compound, may suggest that extracellular 7-OH-MTX levels are >5 times higher in hepatic tissue than in blood. The precise enzymatic mechanism responsible for the 7-hydroxylation of MTX remains to be defined [8], and rabbit data have suggested that a variety of tissues may be responsible for this biotransformation [10]. Our observation may indicate that the metabolite 7-OH-MTX is primarily generated in hepatic tissue in the rat.

In summary, the microdialysis technique can provide reproducible data on extracellular drug exposure in an animal model. This approach for on-line monitoring of extracellular drug levels may have considerable future impact for therapeutic drug monitoring in the clinical setting.

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