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ORIGINAL ARTICLE

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Pharmacokinetics of different doses of methotrexate at steady state by in situ microdialysis in a rat model

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Abstract We used a microdialysis technique to monitor extracellular methotrexate (MTX) levels during the steady state in a rodent model. Microdialysis probes were implanted in the muscle, liver, and kidney of anesthetized male Wistar rats. MTX (18.75–500 mg/kg) was given as a continuous infusion through a venous catheter, and blood samples were obtained through a second venous catheter. Heparinized plasma, ultrafiltered plasma, microdialysis effluent from tissues, and tissue samples (obtained at the end of experiments) were analyzed for MTX content by high-performance liquid chromatography (HPLC). Steady state was demonstrated in the blood and tissues from 2 h until the end of the experiments (6 h). Extracellular drug levels in muscle and liver displayed a linear correlation with doses, whereas kidney levels reached a plateau at an MTX dose of 150 mg/kg per 6 h. Microdialysis-fluid endpoint levels for muscle, liver, and kidney were positively correlated to the endpoint total tissue levels ($r^2 = 0.80, 0.85$, and 0.68, respectively). In the kidneys, the maximal relative tissue MTX accumulation was measured at a total dose of 75 mg/kg per 6 h. At higher doses, the relative drug sequestration declined to less than half of the values observed at this dose. This study demonstrates that the microdialysis technique can provide reproducible data on MTX tissue exposure in an animal model and that it offers a means of serial and reproducible monitoring of extracellular-tissue MTX levels at steady state and over a wide dose range. Pending additional studies, microdialysis may be a helpful technique for elucidating the kinetics of drug delivery to both targeted and toxicity-prone tissues during chemotherapy.

Key words Microdialysis · Methotrexate · Steady state
Rat · Tissues

Introduction

The chemotherapeutic agent methotrexate (MTX) is widely used in the treatment of different types of cancer, such as acute lymphocytic leukemia, osteosarcoma, choriocarcinoma, and breast and head/neck tumors [2, 11]. Solid tumors are composed of heterogeneous tissue, and there is considerable interindividual variation with regard to cellular organization and vascular morphology. This may in part explain the differences in treatment outcome observed after chemotherapy between individuals. Measurements of drug levels in the bloodstream have been routinely employed during MTX treatment for more than a decade [15]. This has been undertaken to facilitate the identification of individuals with impaired drug elimination who are prone to develop serious side effects, but the practice also rests on an assumed relationship between the tumor response and the circulating levels of drug in these patients. However, studies in patients with solid tumors have largely failed to demonstrate firm correlations between MTX blood pharmacokinetics and the chemotherapeutic response [14]. This may in part be due to interindividual heterogeneity in tumor composition.

The monitoring of extracellular tumor drug levels is one of several means by which drug concentrations can be measured in proximity to the biophase. This may be undertaken by using the microdialysis technique [1, 19]. Briefly, microdialysis is performed by inserting

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a tiny semipermeable membrane, which can be flushed with a suitable carrier solution, into the tissues of interest. Low-molecular-weight compounds in the tissue can diffuse across the membrane and into the carrier solution. A constant and low flow in the carrier fluid transports the compounds into suitable collection vials, after which analytical procedures may be undertaken. Compounds of higher molecular weight, such as proteins, are excluded by the dialysis membrane. Due to their small size, the microdialysis probes can be placed in virtually any tissue in vivo without causing extensive structural damage.

In a previous report [8] we have shown that the microdialysis technique is a reproducible method for monitoring MTX pharmacokinetics in plasma and several different tissues in rats after bolus drug injections of 100 mg/kg. Herein we report the results of using the technique during the steady state at several different MTX doses in an otherwise similar non-tumor-bearing rodent model.

Materials and methods

Drugs and chemicals

Unformulated MTX was obtained from Lederle Laboratories (Pearl River, N.Y., USA). The perfusion solution (Ringer acetate; Kabi Pharmacia AS, Halden, Norway) was delivered at ambient temperature by a CMA/100 (CMA Microdialysis AB, Stockholm, Sweden) syringe pump, and the perfusate was collected by a CMA/140 fraction collector. High-performance liquid chromatography (HPLC)-grade methanol was obtained from Rathburn Chemicals Ltd., (Walkerburn, UK). All other reagents were of analytical grade.

In vivo experiments

Male Wistar rats weighing 242 ± 13 g (mean ± 1SD) were obtained from Shaw's Farm (Oxon, UK) and maintained in conventional facilities. The animals had free access to food and water. Experimental procedures did not include hydration and urine alkalinization, which is routinely a part of high-dose MTX regimens in patients. Surgical anesthesia was induced by the subcutaneous administration of 2.0 ml/kg of a mixture of fentanyl (0.05 mg/ml), fluanisone (2.5 mg/ml), and midazolam (1.25 mg/ml). Two polyethylene catheters (PE-10, Clay Adams; Becton & Dickinson, Rutherford, N.J., USA) were inserted into each animal's right jugular vein. One was used for continuous administration of MTX and the other, for delivering maintenance anesthesia by a peristaltic pump (IPN-12; Ismatec SA, Glattbrugg-Zürich, Switzerland). The total infused volume of MTX and anesthetic agent was 0.6–0.7 ml/h. A third catheter (diameter, 1.0 mm; Viggo-Spectramed, Helsingborg, Sweden) was inserted into the left femoral vein and used for blood sampling. Three flexible microdialysis probes (CMA 20; membrane size, 0.5 × 10 mm) were inserted into the cortical part of the right kidney, the adductor muscle of the right femur, and the right liver lobe, respectively. Incisions were closed with Novafil 2–0 (Davis + Geck, Hampshire, UK) sutures. The surgical procedure took approximately 45 min per animal. Subsequently, the rat was put onto a CMA/150 temperature controller set at 38°C. Before the start of experiments the microdialysis probes were flushed with Ringer acetate at 15 µl/min to purge membranes and tubing of air bubbles. The perfusate flow was reduced to 4 µl/min before drug administration and was kept at this rate throughout the subsequent procedures. The MTX doses used were 18.75, 37.5, 75, 150, and 300 mg/kg per 6 h and, in two animals, 500 mg/kg per 4 h.

Blood samples and sample treatment

Samples of dialysis fractions were obtained continuously for 6 h. Blood samples (400 µl) were drawn in the middle of dialysis sample periods at 75, 135, 165, 225, 285, 345, and 360 min and were replaced with an equal amount of Ringer acetate. The blood was transferred to 1.5-ml microcentrifuge tubes containing 14 µl of heparin (5,000 IU/ml; Leo, Ballerup, Denmark) and centrifuged at 5,600 *g* for 2 min. Plasma ultrafiltrates were obtained 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). Preparation of plasma, ultrafiltrates, and dialysis samples prior to chromatography consisted of deproteinization by the addition of a 1:5 vol. of 2 *M* perchloric acid, mixing, centrifugation at 15,000 *g* for 5 min, and transfer of the sample to borosilicate-glass autosampler vials (Chromacol Ltd., London, UK). Tissue samples from kidney, liver, and muscle were obtained at 360 min after drug administration and were stored at − 70°C prior to analysis.

Tissue samples

Samples were cut into pieces weighing 0.3–0.43 g, and a TRIS (10 m*M*, pH 8.0)/$CaCl_2$ (1 m*M*) buffer was added (1:5, w:v). Subsequently, the tissues were homogenized with a Polytron homogenizer (PTA 10 35; Kinematica, Lüzern, Switzerland) for 2 × 15 s. The samples were kept on ice during the procedure. At this point the kidney samples were diluted further (1:10, v:v) with buffer. To 200 µl of homogenate was added 20 µl of proteinase K (10 mg/ml; Boehringer Mannheim, Germany), with subsequent incubation being performed at 65°C for 30 min. Following this, the samples were prepared for analysis as outlined above. Standard curves were constructed by adding known amounts of MTX to homogenized tissue samples. Assay recoveries were quantitative. The standard curves were linear in the 0.2 to 200-µ*M* concentration range ($r^2 = 0.99$), and the assay variability was characterized by a within-run coefficient of variation (CV) of 5% and a between-run CV of less than 10% (data not shown).

Analytical procedure

MTX levels were quantified by a modified isocratic HPLC assay described in detail elsewhere [16]. 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 CT0-6A column oven and an on-line solvent preheater. Samples were injected with a SIL-9A autoinjector maintained at ambient temperature, and MTX was detected by ar SPD-6A V 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; 50 µl of sample was injected. Between analyses the autoinjector line was flushed with a 60% aqueous methanol solution. We did not attempt to quantify MTX metabolites.

Calculations

The MTX area under the time-concentration curve (AUC) was calculated by multiplying the MTX level determined at the termination of infusion by the infusion time. Statistical calculations were performed by least-squares regression analysis.

Results

Steady-state conditions during 6 h of continuous MTX infusions were confirmed by serial bleeds carried out from 75 min onward in the experimental animals. Figure 1 demonstrates the achievement of steady state from 2 h to the end of the sampling period, i.e., at 6 h. Measured steady-state levels suggested a linear relationship with the doses delivered in the 18.75- to 300-mg/kg range. Table 1 gives data on drug protein binding, obtained by analysis of ultrafiltered plasma samples, and AUC estimations in the animals. The table shows that 40%–68% of the MTX in rat plasma is unbound and that the degree of drug binding in plasma decreases with increasing drug concentrations ($r^2 = 0.95$) and confirms a linear correlation between dose and MTX AUC determinations at the different dose levels ($r^2 = 0.99$), with AUC/dose ratios being in the 0.14–0.20 range (Table 1).

During infusions, microdialysis fluid from probes situated in the kidney, liver, and muscle tissues of each animal was collected and analyzed for MTX content. The results show that steady-state conditions in the three tissues were attained by 2 h during the infusions and that dose increments were reflected in quantitatively different ways in the three tissues examined (Fig. 2). This was further substantiated by a plot of tissue microdialysate values against the delivered doses. Figure 3 shows that whereas there was a fairly linear correlation between dose and dialysate drug levels in liver and muscle tissue, the extracellular drug levels in renal tissue reached a plateau at 150 mg/kg per 6 h and a doubling of this dose resulted in virtually identical

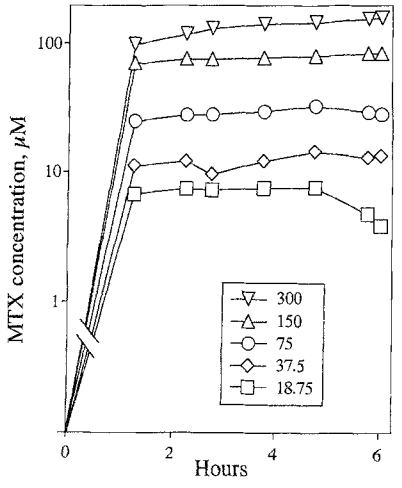

Fig. 1 Total plasma levels of MTX measured during continuous infusions for 6 h at five different doses (18.75, 37.5, 75, 150, and 300 mg/kg per 6 h). Data are given as mean values ($n = 3$). Note the vertical log axis

Table 1 Protein binding and estimated AUC values determined for total plasma MTX levels in rats during steady-state infusions of different MTX doses. Data are given as mean values ($n = 3$)

Dose (mg/kg per 6 h)	Unbound MTX (%)	AUC (m*M* min)	Ratio AUC/dose
18.75	40.7	2.8	0.15
37.5	46.9	5.3	0.14
75	50.8	12.1	0.16
150	57.0	29.3	0.20
300	67.7	53.0	0.18

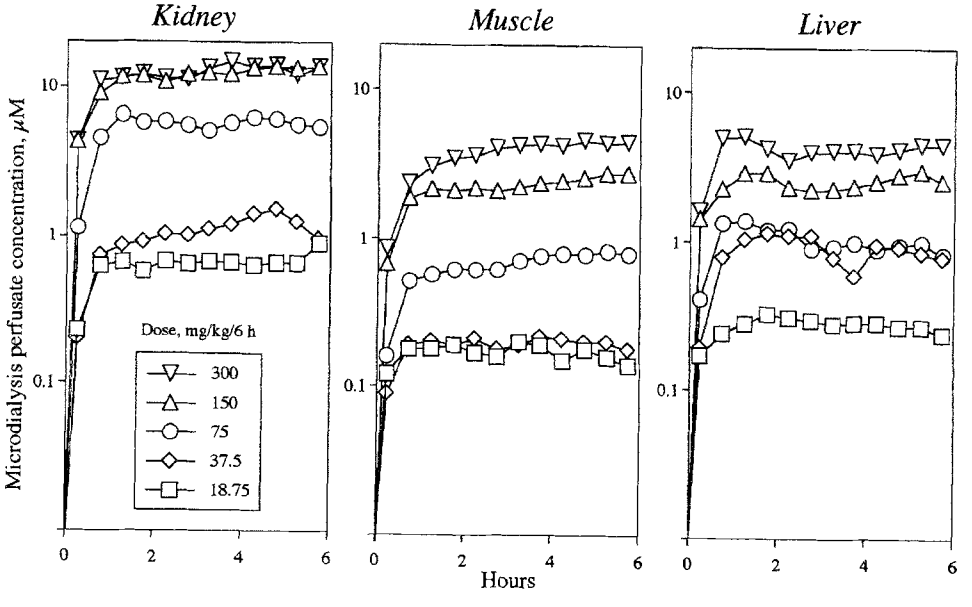

Fig. 2 Microdialysis perfusate levels determined in kidney, muscle, and liver during continuous MTX infusions for 6 h at five different doses (18.75, 37.5, 75, 150, and 300 mg/kg per 6 h). Microdialysis data are not corrected for recovery. Data are given as mean values ($n = 3$). Note the vertical log axis

microdialyzable MTX concentrations. Note should be taken that the dialysates are not corrected for relative recovery [19] and that the reported values thus do not represent the actual extracellular concentrations.

Tissue samples were obtained at the end of the experimental procedures (6 h). The drug levels in tissue samples represent a combination of intracellularly sequestered and extracellular (i.e., microdialyzable) drug. Figure 4 depicts the relationship between the endpoint microdialysate levels and the total tissue levels measured at the same time point. For both hepatic and muscle tissues, these levels were positively interdependent with coefficients of correlation (r^2) of 0.80 and 0.85, respectively. In the renal tissue, the correlation coefficient (r^2) was 0.68 (Fig. 4). The correlations were highly significant ($P < 0.01$).

The drug available for intracellular accumulation should be reflected in the nonbound levels of drug in the blood. Figure 5 represents a plot of the tissue/ultrafiltrable blood drug ratio as a function of the delivered MTX dose. This ratio was constant for all doses examined in liver and muscle tissues. In the

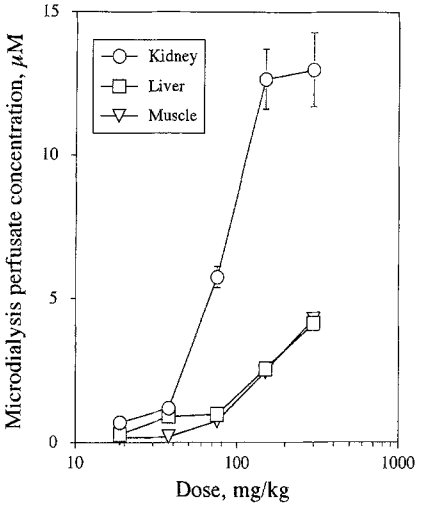

Fig. 3 Relationship between dose and tissue-microdialysis-perfusate plateau levels of MTX achieved during steady state. Microdialysis data are not corrected for recovery. Data are given as mean values ± 1SD ($n = 3$). Note the horizontal log axis

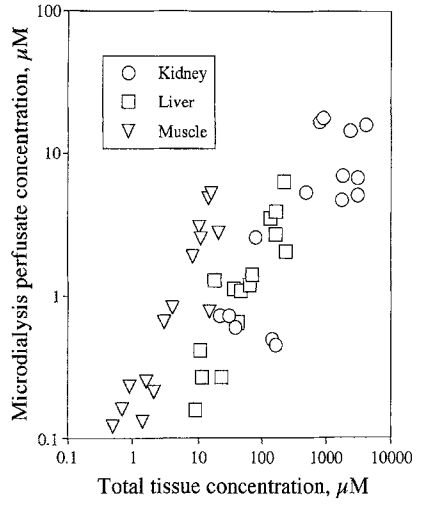

Fig. 4 Relation between total tissue MTX levels and microdialysis perfusate concentrations obtained at the end of experiments, i.e., at 6 h, from rats infused with five different doses (18.75, 37.5, 75, 150, and 300 mg/kg per 6 h) of the drug. Microdialysis data are not corrected for recovery. Note the log scales. The correlation coefficients (r^2) between total tissue MTX levels and concentrations determined in microdialysis fluid from kidney, muscle, and liver were 0.68, 0.85, and 0.80, respectively

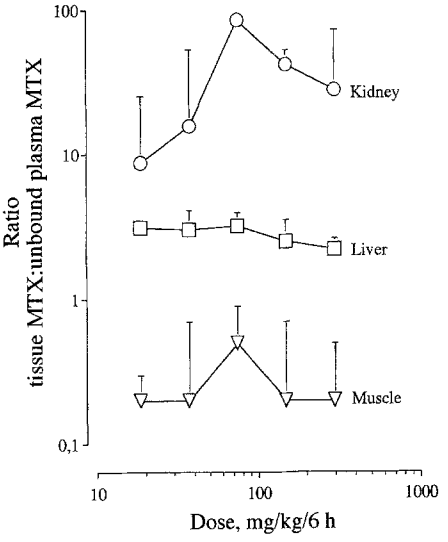

Fig. 5 The total tissue MTX concentration divided by the unbound concentration in the circulation is presented as a ratio and compared for the doses investigated. Data are given as mean values ± 1SD ($n = 3$). Note the log axes

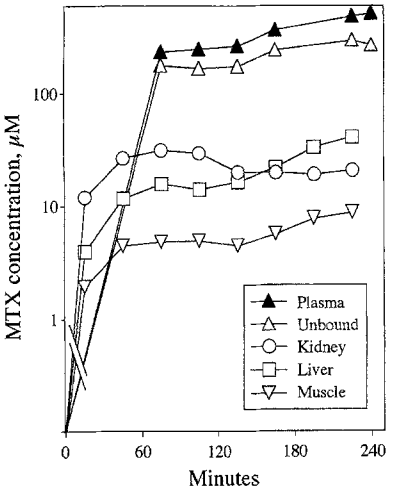

Fig. 6 Levels of MTX measured in one animal that received 500 mg/kg over 4 h. Steady-state conditions were not attained during the infusion. Microdialysis data are not corrected for recovery. Note the vertical log axis

kidneys, the maximal relative tissue MTX accumulation was measured at a total dose of 75 mg/kg per 6 h. At higher doses the relative drug sequestration declined to less than half of the values observed at this dose (Fig. 5).

A few animals were also given MTX doses higher than 300 mg/kg per 6 h. At doses of 500 mg/kg per 4 h, gross interindividual variability between animals was evident. Figure 6 shows data obtained from an experiment in a single animal. It is evident from the figure that steady-state conditions were not reached by 4 h. In addition, high initial kidney dialysate values were followed by a decline in amounts of dialyzable drug, whereas microdialysis-fluid drug levels continued to increase in samples from probes situated in muscle and liver tissue (Fig. 6). Although highly variable, an essentially similar course of events was observed in the other animal given this high MTX dose.

Discussion

MTX is a commonly used and extensively investigated agent in several comprehensive chemotherapy regimens. In the treatment of solid tumors, the drug is commonly used at high doses in combination with the antidote leucovorin [11]. The baseline pharmacokinetic properties and pharmacodynamic effects of the agent have been subjected to numerous studies, and several investigators have correlated bloodstream pharmacokinetic parameters with the clinical outcome after MTX chemotherapy [3, 9]. Notably, these correlations have been evident solely in leukemia patients. In leukemia, blood cells represent at least part of the target tissue for the intended cytotoxicity. Differences between blood and tumor drug levels may, at least to some extent, account for the lack of convincing correlations between pharmacokinetic and -dynamic variables in the therapy of solid tumors such as osteosarcomas [14].

Specific and sufficiently sensitive assays for monitoring MTX are available. Although the drug is photosensitive [6] and adsorbs to glassware [7], our experiments gave no indication that MTX interacted with components of the microdialysis system. Together, these factors constitute the reasons for using MTX as a model compound in the current investigation. MTX doses were selected arbitrarily over a fairly wide range corresponding to doses used in the clinic, including those reported to induce renal or acute hepatic toxicity in rats [4].

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. The microdialysis technique may allow serial sampling from extracellular spaces for monitoring of the tissue penetration of drugs. Such investigations must, however, be based on unequivocal evidence that the technique is capable of providing reproducible data. We have previously presented results suggesting that this is indeed so in non-tumor-bearing rats given single bolus doses of 100 mg/kg MTX [8]. Herein we report the results of microdialysis monitoring of MTX in three tissue compartments in normal rats that were given infusions at doses ranging from 18.75 to 500 mg/kg.

Our plasma data demonstrate that steady-state conditions are reached from 2 h onward during 6-h infusions of MTX in the 18.75- to 300-mg/kg dose range (Fig. 1), that the elimination of MTX from the blood is independent of the delivered dose over the range examined (Fig. 1, Table 1), and that there is a dose-dependent decrease in MTX binding to plasma proteins, possibly reflecting saturation of MTX-binding sites on albumin molecules, with escalating dose (Table 1). Previous experiments with bolus MTX injections in the rat [8] have suggested that 90% of steady-state MTX levels should be reached after approximately 100 min. In human serum, MTX is approximately 50% bound [12, 13], mainly to albumin [17]. Other investigators have reported disproportional elevation of unbound methotrexate levels in human serum at high doses [18]. Since even the highest doses given to the rats are comparable with those used clinically, this observation supports the concept that the protein binding of MTX is subjected to dose-related variability in patients.

In the current study, three different tissue compartments were monitored by the microdialysis technique. The three were selected because they provide considerable differences in structure, morphology, enzymatic activity, and uniformity. The kidneys are the major route for the elimination of MTX, and compromised renal function is one of the most frequent toxicities induced by the compound [10, 11]. Microdialysis data demonstrate that the steady-state conditions found in the bloodstream are reflected in the tissues (Fig. 2) and that the drug displays dose-independent pharmacokinetic properties in muscle and liver tissue but not in renal tissue (Figs. 2, 3). The observed uniformity of plasma-, muscle-, and liver-disposition kinetics is in agreement with observations in rats given bolus MTX doses [8]. In humans, MTX is eliminated through a process of glomerular filtration, reabsorption in the proximal renal tubule and distal tubular secretion [5]. The observed plateau in renal microdialysate MTX levels at the 150- to 300-mg/kg doses may reflect saturation of renal MTX elimination, most probably on the level of tubular secretion.

In tissues the volume of the extracellular compartment is small as compared with that of the intracellular space. MTX is to a large extent transported across cell membranes and sequestered intracellularly. On the assumption that extracellular drug levels are negligible as compared with intracellular MTX concentrations, analysis of tissue homogenates may be used to assess

intracellular MTX levels. In Fig. 4, the tissue MTX concentrations and tissue microdialysate levels have been correlated. A positive correlation over a wide (2-log) concentration range was evident in both liver and muscle. The low degree of correlation found in kidney tissue may reflect difficulties in reproducibly inserting the microdialysis probes, regional differences in MTX levels in this tissue, regional changes in renal tissue because of drug toxicity, drug precipitation in renal tubules, or combinations thereof. A hypothesis of selective renal toxicity is supported by the demonstration of decreased relative cellular MTX uptake at high drug doses.

Figure 5 shows the relationship between unbound plasma MTX levels and intracellular drug concentrations observed over the dose range investigated. Whereas hepatic and muscle cells accumulate a fixed fraction of the free drug irrespective of dose, renal cells incorporate a larger fraction of available drug at lower doses (Fig. 5). The actual extracellular drug levels at which maximal relative influx occurs are difficult to calculate, but ongoing investigations in our laboratory would suggest that microdialysis under the current conditions gives tissue recoveries in the 4%–8% range at a perfusion rate of 4 μl/min. This implies that intracellular MTX transport in the kidney may be facilitated at extracellular concentrations of 100–200 μM. These levels are well above the considerably lower micromolar levels at which passive transport of MTX into cells becomes the dominating mechanism for cellular drug entry [11]. However, a shift in influx kinetics cannot be ruled out as having been responsible for the observed effects (Fig. 5). Also, the data may reflect a gradual cessation of renal function at doses exceeding 100 mg/kg per 6 h. This is supported by observations at even higher MTX doses. In animals given 500 mg/kg per 4 h, steady-state conditions were not observed during infusions, presumably due to compromised renal drug excretion. Data from a single animal (Fig. 6) show a rapid increase in plasma levels during the 1st h, a subsequent plateau of about 1-h duration, and increasing drug levels for the rest of the observation period. Ultrafiltrate MTX concentrations and extracellular levels in liver and muscle tissue closely resemble the total plasma-disposition curve. In renal tissue, extracellular drug levels actually decrease during the final elevations in plasma MTX concentrations (Fig. 6). This picture is consistent with acute MTX-induced renal toxicity. It should be added that this occurs at a renal-tissue MTX concentration of 5.8 m*M*, which is above the solubility for MTX in urine at physiological pH [2, 10].

The data presented herein do not add much to the current understanding of MTX pharmacokinetics. The significance of the results lies in the demonstration that the microdialysis technique offers a means of serial and reproducible monitoring of extracellular-tissue MTX levels at steady state and over a wide dose range. There is a positive correlation between the unbound drug levels measured in plasma and the extracellular concentration of MTX detected in tissues, and the technique is suitable for monitoring the relative changes in MTX levels in these tissues. Positive correlations also exist between the intra- and extracellular levels of MTX measured in liver and muscle tissues. Pending additional studies, microdialysis may be a useful technique for elucidating the kinetics of drug delivery to both targeted and toxicity-prone tissues during chemotherapy.

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