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Determination of extracellular methotrexate tissue levels by microdialysis in a rat model

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Abstract We used a microdialysis technique to determine tissue methotrexate (MTX) levels during steady state in a rodent model. Two different approaches were employed to measure the actual extracellular MTX concentrations in muscle, liver, and kidney tissues of anesthetized Wistar rats. With the reduced-perfusion-rate technique, the flow in the microdialysis perfusate was gradually decreased toward zero to permit calculation of zero-flow intercepts. Using the net change technique, microdialysis probes were perfused with different MTX concentrations to allow an assessment of equilibrium drug levels. For these two methods to be used, drug concentrations in the matrix to be analyzed must remain unchanged during the experimental procedure. In the animal model, steady state was attained after 1.5 h and maintained throughout the rest of the experiments by the administration of MTX as continuous infusions through a venous catheter. In vitro and in vivo, both the reduced-perfusion-rate and net change techniques gave reproducible data that permitted the estimation of extracellular drug concentrations in the dialyzed tissue compartments. The data suggest that the level of unbound MTX in the circulation is fairly similar to the extracellular concentrations in the muscle and liver. In the kidney, MTX levels were measured to be 3–8 times higher than those of unbound, circulating MTX, and a considerable discrepancy between the two methods used for estimations was apparent. These results demonstrate that both the net change and reduced-flow microdialysis techniques can produce reproducible and precise data. The results may constitute a basis for determining recoveries and, thus, true extracellular drug levels during in vivo microdialysis of MTX. This may be of importance in delineation of the relationship between tissue MTX levels and outcome in a variety of normally inaccessible compartments during cancer pharmacotherapy.

Key words Microdialysis · Methotrexate · Tissue · Recovery · Rat

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Introduction

The antifolate methotrexate (MTX) has demonstrated antineoplastic activity in the treatment of several different types of cancers [4, 13]. At least in part, therapeutic efficacy is related to the extent of intracellular MTX polyglutamation [1, 8, 15]. MTX polyglutamates are retained intracellularly for longer periods [12, 14], have higher affinity for the target enzymes [2, 22], and may also act qualitatively differently [7] as compared with the parent compound. Glutamation of MTX is concentration- and time-dependent in the sense that it requires exposure to micromolar levels of extracellular drug for prolonged periods [11]. The maintenance of adequate extracellular tissue levels of the drug may thus be crucial to MTX-induced cytotoxicity.

In previous microdialysis studies in a non-tumor-bearing rodent model, we have found positive correlations between the MTX levels in plasma and the relative extracellular levels in the muscle, liver, and kidney [9, 10]. The present study was focused on techniques to determine the true levels of MTX in these tissues by microdialysis.

There are several means by which microdialysis can be used to determine the actual extracellular concentration of a compound [3, 26]. Quantitative microdialysis together with mathematical models can be applied for the estimation of diffusion kinetics and thus permit an assessment of drug levels in tissues [20]. However, empiric studies can also provide data for estimating extracellular levels of the compound of interest. One approach is to use a suitable internal standard to calculate the relative recoveries and thus the extracellular concentrations of the compound [16]. Second, the relationship between the perfusion rate and the relative recovery can be exploited. In the microdialysis system, the perfusion rate is linearly correlated to the inverse of the perfusate concentration. A gradual reduction in perfusion rate with measurements of perfusate concentrations makes it possible to interpolate a zero-flow/quantitative recovery perfusate value for a particular substance [25, 26]. Third, the equilibrium concentration (i.e., where no net transport of compound across the dialysis membrane takes place) can be calculated by the addition of known levels of the compound of interest to the perfusion medium and assessment of the concentration changes in the perfusate [19, 25]. The second and third techniques are dependent on steady-state levels of compound at the site of microdialysis.

In the present study we investigated the possibility of calculating relative recoveries and thus assessing actual extracellular MTX concentrations by the two latter microdialysis techniques in vitro and in vivo.

Materials and methods

In vitro experiments

Assessment of relative MTX recoveries in Ringer acetate and plasma was carried out as outlined previously [9]. Reduced-perfusion-rate (RPR) and net change (NC) technique microdialysis experiments were performed with probes placed in Eppendorf tubes containing the samples to be dialyzed. In both series, the samples contained either 20 or 90 μM MTX. The tubes were kept at 37° C for the duration of experimental procedures. Ringer acetate was used as the carrier solution and for dissolving MTX in the samples. In the NC technique experiments, MTX was added to the perfusion solutions to final concentrations of 10, 60, and 110 μ*M*.

In vivo experiments

Male Wistar rats weighing 244 ± 14 g (mean ± 1SD) were obtained from Shaw's Farm (Oxon, UK) and maintained in conventional facilities with free access to food and water. Animals were anesthetized by a subcutaneous injection of a 2.0 to 2.5-ml/kg/dose of a mixture of fentanyl (0.05 mg/ml), fluanisone (2.5 mg/ml), and midazolam (1.25 mg/ml). The surgical procedures were carried out as described elsewhere [10]. In some experiments an additional catheter (PE-50; Clay Adams, Becton Dickinson, Rutherford, N.J., USA) was inserted in the bladder for urine collection. Before the start of experiments, the microdialysis probes were flushed with Ringer acetate at 15 μl/min to purge the membranes and tubing of air bubbles. Before drug administration the perfusion rate was reduced to 4 μl/min for the RPR experiments and to 1 μl/min for the NC experiments. In the latter experiments the same MTX concentrations were used in perfusion solutions as during in vitro procedures. Groups of animals ($n = 3$) to be used for either RPR or NC experiments were given 75 mg/kg MTX over 6 h as a continuous infusion. Other groups ($n = 3$) were given MTX doses ranging from 18.75 to 300 mg/kg over 6 h.

Samples, sample treatment and analysis

Samples of dialysis fractions were obtained continuously for up to 6 h. During in vivo procedures, blood (400 μl) and urine samples were obtained in the middle of dialysis periods at 75, 135, 165, 210, 300, and 360 min, and the drawn blood was replaced with an equal volume of Ringer acetate. Plasma ultrafiltrates were produced by centrifugation of plasma samples at 3,600 *g* for 30 min through a 30-kDa cutoff filter (Ultrafree MC, Millipore, Bedford, Mass. USA). Detailed descriptions of sample procurement and treatment procedures are given elsewhere [9, 10]. MTX analyses were undertaken by a modified isocratic high-pressure liquid chromatography (HPLC) assay described in detail elsewhere [23] and modified as previously described [9, 10].

Calculations

All regression lines were calculated by least-squares analysis. In vitro relative recovery was calculated by comparison of the concentration in the dialyzed sample with that in the microdialysis perfusate. In the RPR experiments the regression lines were interpolated to a flow rate of zero, and in the NC procedures the tissue levels were estimated by construction of regression lines through the abscissa corresponding to equilibrium. In vivo relative recovery was estimated by comparison of microdialysis perfusate values from the tissues with the estimated extracellular tissue values obtained by either of these two methods.

Results

The relationship between the perfusion rate and the relative recovery of MTX by microdialysis in vitro is depicted in Fig. 1. A gradually diminished microdialysis flow rate resulted in an increased recovery of MTX. At the lowest perfusion rate (0.25 μl/min) this recovery was slightly higher than 90%. Plotting of the inverse perfusate concentration against the perfusion rate gave estimated target concentrations of 17.2 and 90.9 μ*M* MTX, which corresponded closely to the actual sample values of 20 and 90 μ*M* (Fig. 1, upper right).

Estimations of MTX concentrations by the NC technique in vitro were undertaken at two MTX concentrations. Microdialysis probes were perfused with Ringer acetate spiked to three different MTX concentrations. Net changes in perfusates were plotted against the MTX concentration in the afferent part of the microdialysis system. Regression analyses permitted the estimation of interception points where net changes were zero. These concentrations (19.9 and 95.0 μ*M*) were very close to the MTX levels in the dialyzed samples, i.e., 20 and 90 μ*M* (Fig. 2). Regression analysis demonstrated linearity ($r^2 = 0.96$) in both experiments.

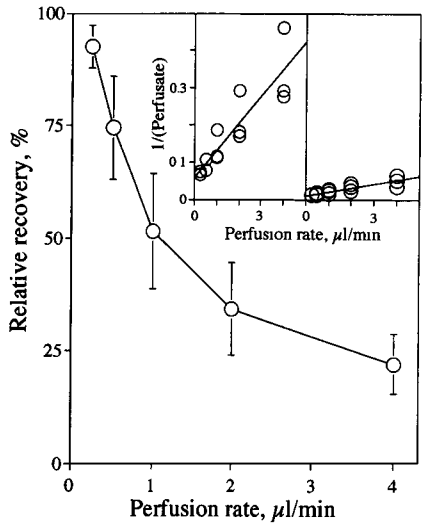

Fig. 1 In vitro relationship between the relative MTX recovery and the perfusion rate. Samples to be dialyzed contained 20 or 90 μ*M* MTX in Ringer acetate, which was also used as the perfusion fluid. Data are given as mean values ±1SD (n=6). In the *upper right corner*, the linear relationship between the reduced perfusion rate and the inverse of the perfusate concentration is depicted. The regression lines intercepted at 17.2 and 90.9μ*M*

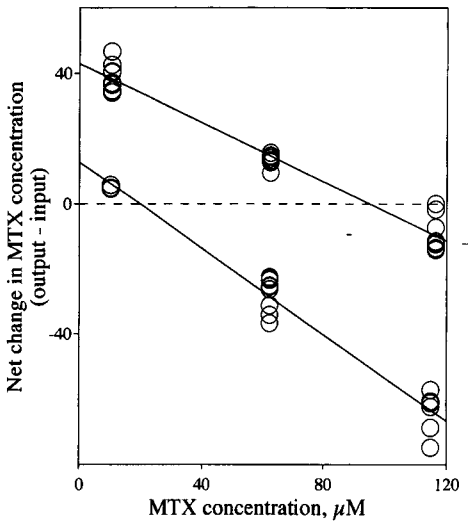

Fig. 2 In vitro estimation of MTX concentrations in samples containing 20 or 90 μ*M* MTX in Ringer acetate. The microdialysis fluid was Ringer acetate to which MTX was added to final concentrations of 10, 60, or 110 μ*M*. The regression lines where the net change is zero intercept at MTX concentrations of 19.9 and 95.0 μ*M*. Data points from two different experiments are given.

Steady state during the latter part of infusions of 75 mg/kg MTX over 6 h in the experimental animals is shown in Fig. 3. Total and unbound MTX levels did not change during the 1.5 to 6-h interval after the start of the infusion, and the rate of renal MTX elimination also remained unchanged during this time span. An average of 21% of the delivered dose of MTX was recovered in the urine during the infusions (Fig. 3).

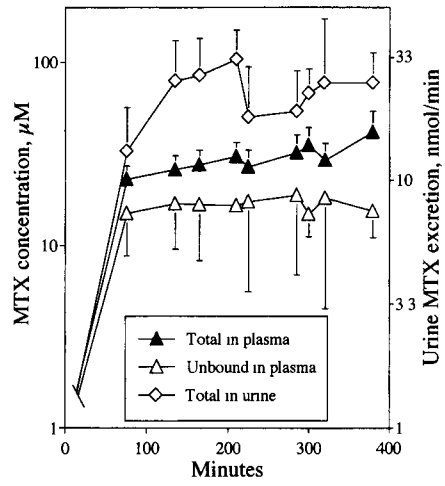

Fig. 3 Total and unbound plasma levels and urinary concentrations of MTX measured during continuous infusions of 75 mg/kg of the drug over 6 h. Data are given as mean values ±1SD (n = 6). Note the vertical log axis

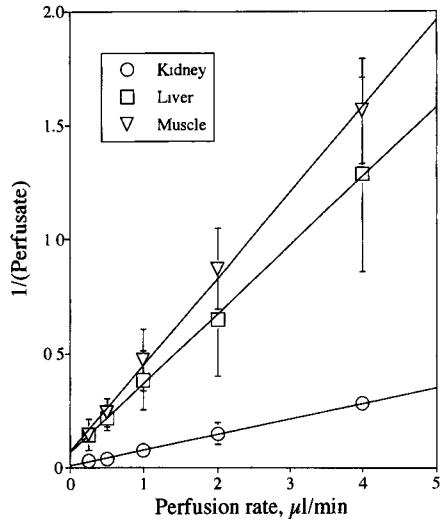

Fig. 4 In vivo linear relationship between the inverse of microdialysis perfusate levels and the reduction in the perfusion rate. Intercepts for regression lines (i.e., zero flow) were at 13.8, 15.1, and 105.1 μ*M* MTX in muscle, liver, and kidney, respectively. Correlation coefficients (r^2) for regression lines, calculated from the pooled data, were 0.99. Data are given as mean values ± 1SD (n=3)

For evaluation of the RPR technique in vivo, zero flow in the dialysis system was approximated by gradual flow reductions to the minimum that was practically feasible (0.25 μl/min). In Fig. 4 the inverse of muscle, liver, and kidney perfusate values from three animals are plotted against the perfusion rate. Correlation coefficients (r^2) were 0.99 for all three regression lines.

Figure 5 depicts the results obtained in a NC-technique experiment in a single, representative animal. At 90 min after the commencement of the MTX infusion

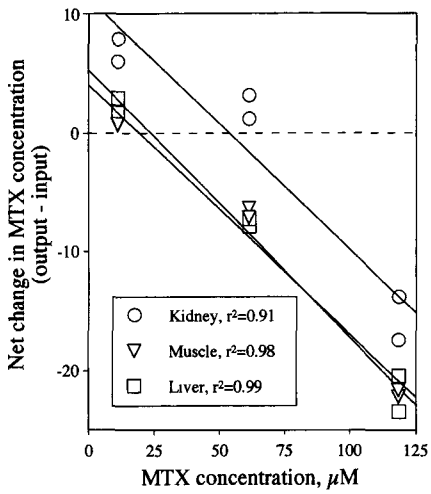

Fig. 5 In vivo estimations of extracellular MTX levels in muscle, liver, and kidney by the NC microdialysis technique during steady state after the administration of 75 mg/kg MTX over 6 h. The perfusion fluid was identical to that used in vitro (see Fig. 2). Data are given as single points for each concentration and tissue compartment

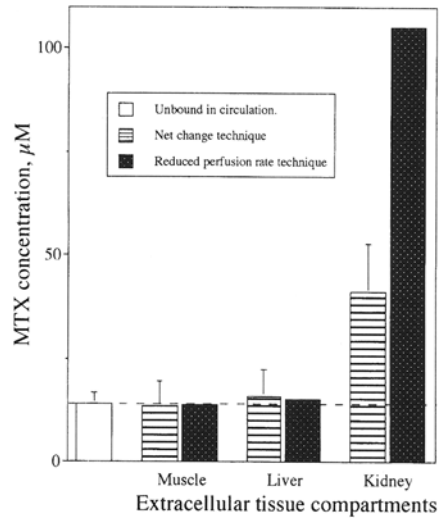

Fig. 6 Extracellular MTX levels in muscle, liver, and kidney as estimated by the NC (*hatched bars*) and RPR (*black bars*) techniques and compared with unbound ($n=6$) MTX in the circulation. Microdialysis data are given as mean values $\pm$1SD ($n=3$)

(i.e., at steady state), microdialysis probes situated in the tissues were perfused (1 μl/min) with Ringer acetate containing MTX at concentrations of 10, 60, and 110 μ*M*. Perfusion with each concentration lasted for approximately 1 h, and the three different concentrations were introduced in random order. Analysis of the MTX content in the perfusion medium both before and after dialysis permitted the construction of regression lines (r^2, 0.91–0.99) that intercepted the equilibrium line at the assumed extracellular tissue MTX levels (Fig. 5).

The unbound fraction of MTX in the circulation at steady state (1.5–6 h) was compared with the extracellular levels estimated in muscle, liver and kidney tissues as outlined above (Figs. 4, 5). The results are shown in Fig. 6. The hatched bars represent estimations done by the NC technique. Regression analyses (all sites and animals) showed correlation coefficients (r^2) in the 0.72–0.99 range. The black bars represent zero-flow intercepts of regression lines established by the RPR method. As is evident from the figure, estimated extracellular muscle and liver MTX levels were similar to the unbound drug levels in the blood, whereas the corresponding levels in kidney tissue were 3–8 times the unbound MTX concentration in the circulation, with the RPR technique giving the higher values.

Table 1 summarizes recovery data obtained for MTX in the different matrices examined. All recovery data relate to one type of microdialysis probe and to one perfusion rate. The relative recovery of MTX was >20% in Ringer acetate and considerably lower (<10%) in extracellular kidney, liver, and muscle compartments. Recovery from Ringer acetate was very similar to that from plasma when the latter was corrected for protein binding of the drug (Table 1).

Table 1 MTX recoveries recorded after both in vitro and in vivo microdialysis undertaken as outlined in Materials and methods. The measured recoveries are peculiar to the system employed (probes CMA 20, 10 mm; Ringer acetate dialysis fluid; flow, 4 μl/min)

	In vitro			In vivo		
	Mean (%)	1 SD	*n*	Mean (%)	1 SD	*n*
Ringer acetate	21.8	1.3	9			
Plasma	18.0	3.1	9	14.9	4.6	14
Unbound in plasma	22.7	3.1	9	18.1	4.6	14
NC technique:						
Extracellular space in kidney				8.2		3
Extracellular space in liver				5.5		3
Extracellular space in muscle				4.4		3
RPR technique:						
Extracellular space in kidney				3.2		3
Extracellular space in liver				5.7		3
Extracellular space in muscle				4.3		3

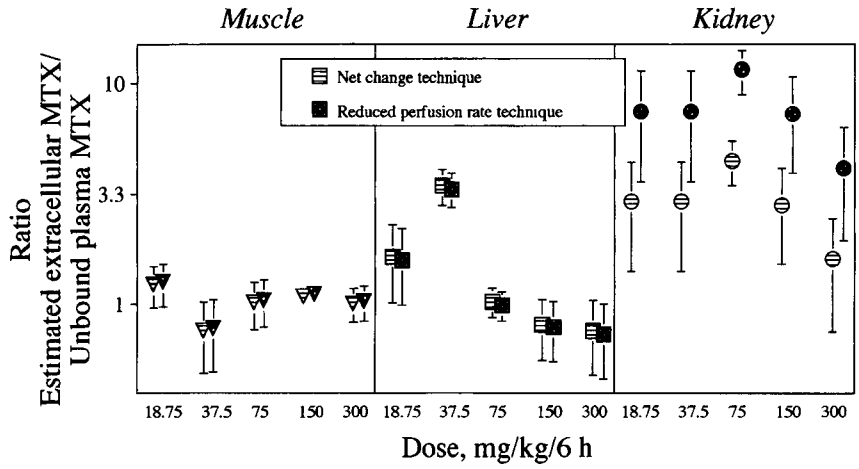

Fig. 7 Ratio between extracellular tissue and circulating unbound MTX in groups of rats given 18.75, 37.5, 75, 150, or 300 mg/kg MTX as 6-h continuous intravenous infusions. Estimations of extracellular MTX concentrations were undertaken by correcting observed levels for recoveries obtained in separate experiments. The measured recoveries are peculiar to the system employed (probes CMA 20, 10 mm; Ringer acetate dialysis fluid; flow, 4 µl/min). Data are given as mean values ± 1SD ($n=3$)

Ratios between extracellular tissue MTX and unbound, circulating drug, estimated by correction of microdialysate levels for recoveries as determined by both methods, were calculated in femoral muscle, liver, and renal cortical compartments in groups ($n=3$) of rats that had been given MTX in the 18.75 to 300-mg/kg dose range as 6-h infusions. This rests on the assumption that relative recovery is constant over the dose range examined. In both the muscle and the liver, ratios were in the 0.8–3.5 (mean values) range for all dose levels examined. In the renal tissue, estimated extracellular MTX concentrations were ≥2.8 times the unbound blood concentrations over the entire dose range tested (Fig. 7).

Discussion

A central tenet to drug monitoring in oncology is the assumed relationship between cytotoxicity and drug concentration in tumor tissue. Determinations of drug levels in solid cancers are generally not undertaken, and tumor biopsy procedures rarely, if ever, permit serial sampling from tissues of interest. Using in vivo systems with the antifolate MTX as the model compound, we have previously demonstrated that the microdialysis technique produces reproducible pharmacokinetic data [9] and can be used to investigate phenomena such as dose-dependent distribution kinetics [10]. These investigations have not included the determination of actual extracellular drug levels.

The in vitro part of the present study shows that estimations of MTX levels by both the RPR technique (Fig. 1) and the NC method (Fig. 2) are feasible. The utility of the former has been demonstrated by other investigators both in vitro and in vivo for substances such as theophylline [25], glucose [5, 18], and adenosine [19]. To our knowledge, the present study is the first in which quantitation of extracellular levels of an anticancer drug by microdialysis has been attempted. In the RPR experiments, the relative recovery of MTX increased in a nonlinear manner with the decline in perfusion rate. Both methodologies determined the MTX levels in dialyzed fluid with <15% variation from the actual concentrations. However, the experimental conditions, including microdialysis of a liquid with no drug-binding capacity or diffusion-inhibiting barrier, must be assumed to be very different from those encountered in vivo.

During the in vitro estimations, the reduction in MTX concentration in the dialyzed samples due to passage over the microdialysis membrane was negligible. In vivo, the closest correlate to this condition is the steady state. The steady-state levels (Fig. 3) correspond well with previous findings in this animal model [10]. Extracellular drug levels in the muscle, liver, and kidney compartments remained virtually unchanged from 1.5 h onward. Steady state is a prerequisite for using either the NC or the RPR technique in vivo [26].

Microdialysis data obtained in vivo (Figs. 4–6) show that the extracellular muscle and liver MTX concentrations in the rat are very similar to the unbound drug levels in the circulation (Fig. 6). Since MTX is soluble at the concentrations measured and has a volume of distribution that approximates 1 l/kg [24], an even distribution of unbound drug between the bloodstream and the extracellular spaces in well-perfused organs with no defined diffusion barrier for small molecules is to be expected. In renal tissue, estimated extracellular levels were 3–8 times higher than the concentration of unbound drug in plasma (Fig. 6). This reflects that the kidney constitutes the major excretory route for MTX, which converges to this organ through the action of several mechanisms, including glomerular filtration and tubular secretion [6, 11, 13].

Not surprisingly, the recovery data obtained for MTX under different experimental conditions (Table 1)

show that a larger fraction of drug is recovered from liquid than from solid matrices. This is explained by the influence of limiting diffusion factors such as tortuosity in solid tissues and by the limited volume fraction of the extracellular space [21]. In brain tissue, the diffusion rate of endogenous substances of low molecular weight is reported to be at least 2.5-fold slower than that in a liquid matrix [17]. This corresponds well with the differences in recoveries evidenced by comparison of Ringer acetate or plasma with solid tissues in the present experiments (Table 1). Relative recoveries based on the extracellular levels estimated by the two methods employed were almost identical in liver and muscle tissues but differed by a factor of 2.6 in the kidney. The different results obtained by the two methodologies may be due to several factors, among which tissue heterogeneity, variation in probe placement, and differences in urine production may contribute significantly. These problems are at least in part compounded by the small size of the animals used and may be overcome by studying larger animals or undertaking clinical evaluations. Another likely possibility is that one of the methods may have significant advantages over the other for this type of determination. If this is indeed so, the present data give no foundation for determining which of the techniques is the better.

It should be noted that estimation of extracellular MTX levels by microdialysis in the three compartments does not take into account drug binding to components of the extracellular matrix or cellular surfaces. Thus, estimates of extracellular MTX concentrations in tissues reflect solely the unbound fraction of the drug.

Previous in vitro data from our laboratory suggest that microdialysis recoveries of MTX are not concentration-dependent [9]. We therefore attempted to apply estimates of actual extracellular MTX tissue levels in a model in which rats were exposed to drug infusions at different doses. Figure 7 shows the ratio between estimated extracellular levels and unbound MTX in plasma in the different tissue compartments. The data show recoveries obtained by both the NC and the RPR method. In muscle and liver tissues the two techniques gave similar results and estimated levels that closely approximated the unbound MTX levels in plasma over the entire dose range tested. As stated above, estimates of extracellular drug in the kidney are to a large extent dependent on which of the two methods is employed, and neither technique calculates tissue levels resembling unbound plasma MTX concentrations (Figs. 6, 7). The estimated extracellular kidney levels were found to be approximately 2 times higher than unbound plasma levels when calculations were based on NC data and 5.5 higher when RPR-derived data were employed. We must therefore assume that the actual extracellular MTX levels in kidney tissue are elevated 2.0 to 5.5-fold as compared with unbound plasma concentrations at steady state. Application of the NC and RPR methodologies results in estimations that appear to be relatively precise and are reproducible over a wide concentration range. The reason for the discrepancy between the two methods for kidney estimations remains obscure.

In summary, extracellular tissue concentrations of MTX were assessed by microdialysis in a rat model. Two techniques were evaluated: the NC and the RPR method. The two methods gave similar results when applied in vitro and in the animal model, and reproducible data were obtained under a variety of conditions, including dialysis of muscle and liver tissues. However, analysis of data obtained after microdialysis of kidney tissue revealed considerable differences between the two methods, with the RPR approach giving 2.6-fold higher estimates as compared with the NC method.

Herein we show that implementation of these techniques permits, with some limitations, the estimation of extracellular drug levels in tissues. The results would seem to suggest that microdialysis may be a useful addition to current methodologies employed in both preclinical and clinical evaluation of anticancer agents.

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