Determination of Free Extracellular Levels of Methotrexate by Microdialysis in Muscle and Solid Tumor of the Rabbit

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Purpose. To determine of the pharmacokinetic profile of methotrexate (MTX) in blood and extracellular fluid (ECF) of VX2 tumor and muscle in rabbits.

Methods. Microdialysis probes were inserted into VX2 tumor and in muscle tissue. Following intravenous administration of MTX (30 mg/kg), serial collection of arterial blood samples and dialysates of muscle and tumor ECF for 4 h was carried out. Quantitation of MTX and determination of free plasma concentrations was performed by fluorescence polarization immunoassay and ultrafiltration, respectively. Correlations were established between the unbound plasma and ECF MTX concentrations.

Results. Total and free plasma concentrations exhibited a parallel three exponential decay in both healthy and tumorigenic animals. Total clearance (8.9 *vs* 6.5 $ml^{-1}.min^{-1}.kg^{-1}$) and volume of distribution (4.0 *vs* 2.9 $l.kg^{-1}$), however, tended to decrease in the tumor-bearing group. The ECF/plasma AUC ratio equaled 14.2 ± 8.8% in muscle and 23.9 ± 15.9% in tumor. The concentration-time profile of muscle ECF MTX was parallel and highly correlated (r = 0.97) to that determined in plasma. In contrast, free MTX plasma levels were not correlated with tumor ECF concentrations (r = 0.564).

Conclusions. In addition to the well-known pharmacological variability in the concentration-effect relationship, the important inter-individual variability in tumor exposure to MTX may partly explain that studies in patients with solid tumors have often failed to demonstrate firm correlations between MTX blood pharmacokinetics and the chemotherapeutic response.

KEY WORDS: microdialysis; methotrexate; muscle; VX2 carcinoma; rabbit.

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ABBREVIATIONS: ECF, extracellular fluid; MTX, methotrexate; HBSS, hank's balanced salt solution; C_{in}, MTX concentration of the microdialysis perfusate; C_{out}, MTX concentration of in the microdialysis dialysat samples; C_{max}, maximum drug concentration; AUC, area under concentration curve for plasma drug concentration from time 0 to infinity; AUC_{free}, area under concentration curve for unbound plasma concentration from time 0 to infinity; $AUC_{M\text{-}ECF}$, area under concentration curve for muscle unbound ECF drug concentration from time 0 to infinity; $AUC_{T\text{-}ECF}$, area under concentration curve for tumor unbound ECF drug concentration from time 0 to infinity.

INTRODUCTION

Today, with a few exceptions, drug monitoring in oncology is still limited to the prevention of side effects in patients with impaired drug elimination and does not allow one to predict the treatment response in the individual patient. The lack of analytically guided drug therapy is related to the many problems encountered in using blood pharmacokinetic data to predict concentration at the site of action, as evidenced by studies in patients with solid tumors which fail to demonstrate firm correlations between methotrexate (MTX) blood pharmacokinetics and the chemotherapeutic response (1). This fact is due to factors peculiar to tumor tissue. Indeed, most solid tumors present a difficult target for many antineoplastic agents due to their limited blood supply, which has often been found to be lower than that of the organ of origin, and the existence of the blood-tumor barrier. Moreover, the highly variable tissular level of the drug depends on many factors such as localization, size, and type of the tumor. Solid tumors are composed of heteregeneous tissue exhibiting inter-individual differences in terms of cellular organization and vascular morphology.

Consequently, a clear understanding of the targeting and disposition of chemotherapeutic agents into the tumor could be of help in elucidating the complex relationship between drug levels at the sites of pharmacological action and both blood concentrations and response to cytotoxic agents. However, determinations of drug levels in solid tumors are generally not undertaken and tumor biopsy does not permit serial sampling thus explaining the paucity of data in the literature.

Unbound drug concentration in tissue extracellular fluid (ECF) is known to be a determinant for pharmacological drug effects in the body. In this regard, microdialysis can provide direct access to extracellular space for monitoring the unbound drug concentrations in the vicinity of the biophase and this technique has been successfully applied to determine the tissue exposure to MTX in healthy rats (2–5).

Therefore, the purpose of the present study was to clarify the pharmacokinetic profile of the free fraction of MTX, used as a model compound, in ECF of VX2 tumor implanted in the thigh of rabbits. After a bolus intravenous injection of the drug, blood concentration-time profiles were established, and concentration-time profiles in ECF of tumor and muscle were determined by microdialysis. Correlation coefficients between plasma and ECF drug concentrations in muscle and tumor were calculated.

MATERIALS AND METHODS

Drugs and Chemicals

MTX was obtained from Laboratoires Lederlé (Oullins, France). Purified water was obtained from an Alpha-Q water purification system (Millipore, Saint-Quentin en Yvelines, France). All other chemicals were reagent grade or better.

Microdialysis System

Experiments were carried out with a CMA/100 microinjection pump (Phymep, Paris, France). A CMA/20 flexible microdialysis probe with a membrane length of 10 mm (Phymep,

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Paris, France) was used for sampling in both tumor and muscle tissue. The probe membrane had a tip diameter of 500 μm and a 20,000 Daltons nominal cutoff. Samples were collected using a CMA/140 microfraction collector (Phymep, Paris, France).

Animals

Ten male New-Zealand white rabbits (Elevage Scientifique des Dombes, Châtillon sur Chalaronne, France) weighing between 2 and 3 kg were used. They were housed in individual cages and left to acclimatize for one week in conventional facilities. Animals received food (Type 112, UAR, Epinay, France) and tap water *ad libitum.*

Tumors

The VX2 cells were kindly provided by Institut Gustave Roussy (Villejuif, France). Cell suspensions were prepared in sterile Hank's balanced salt solution (HBSS: 136.89 mM NaCl, 5.37 mM KCl, 0.83 mM $MgSO_4$, 0.34 mM Na_2HPO_4, 1.68 mM $CaCl_2$, 4.17 mM $NaHCO_3$, 0.44 mM KH_2PO_4, 1.05 mM $MgCl_2$, 4.5 mM glucose). After washing the VX2 cells three times in HBSS, the suspension of viable cells was counted by trypan blue dye exclusion. Three milliliters of the cell suspension in HBSS at 2.10^7 viable cells per milliliter were injected intramuscularly into the lateral thigh muscle of rabbits. Tumor-bearing animals were kept for at least one month after implantation until the tumor had reached a diameter of 3–4 cm.

Microdialysis Probe Calibration

In order to assess the feasibility of the dialysis probe in picking up MTX, *in vitro* recovery was determined by placing a microdialysis probe in HBSS spiked with MTX to obtain a concentration of 70 μM. Dialysis probes were perfused with HBSS at a flow rate of 7 μl/min and at 37°C. The recovery was defined as the ratio between the concentration of MTX in the outflow solution and the concentration of MTX in solution outside the probe. Eight dialysate samples were collected over 15 minute intervals for each probe.

In vivo recovery was determined by retrodialysis. After implantation in muscle or in tumor tissue, as described in the surgical procedure, the probes were perfused at a flow rate of 7 μl/min with HBSS containing MTX (22 μM). MTX concentrations were determined in the dialysate samples every 15 min for 4 hours (C_{out}). The recovery was determined from the ratio of the concentration lost to the initial concentration in the perfusate (C_{in}):

$$\text{Recovery}_{\text{in vivo}} = (C_{in} - C_{out})/C_{in}) \times 100$$

Surgical procedure. All animal procedures adhered to the Principles of Laboratory Animal Care (NIH publication, 1985). Rabbits were anesthetized with ethyl carbamate (1.25 mg/kg). The animals remained anesthetized for the entire duration of the experiment. The right femoral artery was used for blood collection via an indwelling catheter (polyethylene catheter with an inner diameter of 1.19 and an outer diameter of 1.70 mm, (Biotrol, Paris, France). The exposed artery and catheter were irrigated with heparinized saline after inserting the catheter. After a skin incision, the microdialysis probe was inserted toward the center of the tumor at a depth of 1 cm corresponding to the length of the probe or into the healthy muscle tissue corresponding to the site of tumor growth with the help of an introducer (diameter: 550 μm). At the end the experiment, animals were sacrificed, the whole tumor was extracted and a dissection was performed in order to verify the placement of the probe in the tumor tissue.

Pharmacokinetics Experiments

Two groups of healthy and tumor-bearing rabbits received an i.v. bolus injection of MTX (30 mg/kg; 25 mg/ml) *via* the marginal ear vein at a rate of 1 ml/min. The animal's body temperature was recorded by means of a Thermalert TH-5 controller (Physitemp, Clifton, U.S.A.).

Blood samples (1.5 ml) were collected from the right femoral artery immediately prior to dosing and at predetermined time-points (2.5, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240 min) after drug administration. The catheter was flushed with heparinized saline (25000 UI/l, Héparine Choay, Paris, France) after each sampling. Blood was harvested into heparinized tubes and centrifuged immediately after collection. The plasma was separated, frozen, and stored at −20°C until assayed.

After insertion, the microdialysis probes were flushed with HBSS to purge the membranes and tubing of air bubbles, and were allowed to equilibrate inside the tissue for 30 min before drug administration in order to ensure a constant *in vivo* recovery (see fig. 3). After correction for dead volume, the dialysate was continuously collected every 15 min for 4 h.

Analytical Procedure

Plasma samples and dialysates were directly analyzed without prior cleanup. MTX concentrations were determined on an Abbott TDx automated fluorescence polarization analyzer (Abbott, Rungis, France) operating on the principle of fluorescence polarization immunoassay. This analytical method is specific to MTX and is not interferred with by its major metabolite, 7-hydroxymethotrexate (6).

Plasma Protein Binding and Serum Protein Electrophoresis

The protein binding was determined by ultrafiltration. Five hundred microliters of plasma were placed in an MPS-1 micropartition system (Amicon, Epernon, France) equipped with a YM-T membrane filter. Centrifugation was performed at 2000 g for 20 min. The filtrate was then used directly to determine the free unbound concentration of MTX in plasma. The percentage of free drug was calculated as the ratio between area under the concentration curve for free plasma concentration and area under the concentration curve for total plasma concentration: $(AUC_{free}/AUC_{total}) \times 100$.

The total concentration of serum proteins was determined according to the Biuret reaction by using a BM/Hitachi 717 911 automatic analyzer (Boehringer Mannheim, Meylan, France). Serum proteins were separated by electrophoresis on a cellulose acetate membrane into albumin, α-globulins, β-globulins and γ-globulins. The proportion of each serum protein was determined by scanning the electropherograms stained with Ponceau red in a Sebia Preference densitometer (Sebia, Issy-les-Moulineaux, France).

Data Analysis

Concentrations of MTX versus time were plotted on semi-logarithmic graphs. Plasma and ECF concentration-time data were fitted to a polyexponential equation with first-order elimination kinetics by nonlinear least-squares regression (Rstrip, MicroMath Inc, Salt Lake City, U.S.A.). The area under the curve was calculated from time 0 to infinity. The total clearance was calculated by dividing the dose by the AUC. The terminal half life ($t_{1/2}\lambda_z$) was calculated as In $2/\lambda_z$ where λ_z was the smallest elimination rate constant. The apparent volume of distribution was obtained by dividing the total clearance by λ_z. The results of the kinetic analysis are expressed as mean ± S.D.

ECF concentrations of MTX were determined by correcting the dialysate concentration by the *in vivo* recovery. Since microdialysate concentrations are time averaged over the collection interval, these values were considered as being equivalent to the actual concentration at the middle point of the time interval.

Statistical calculations were carried out on a microcomputer with Statgraphics Plus (Version 1, Manugistics, Rockville, U.S.A.). A Mann-Whitney (Wilcoxon) W test was used to compare the difference between groups. Non-parametric tests were used because the number of animals was small. Probability values of <0.05 were considered to be significant.

RESULTS

Clinical and Biological Findings

Following tumor implantation, the activity of the animals progressively declined, possibly due to the growth of the tumor in the thigh. After the tumor bearing animals gained weight during the first eighteen days, they exhibited a progressive loss of body weight (about 11% when compared to the control group at day 28) without any other apparent clinical signs. In the control group, the serum albumin concentration and the levels of globulins remained unchanged (Table I) within the 28 day study period. In contrast, the serum albumin concentration decreased significantly, and the concentrations of α, β and γ-globulins increased significantly in the tumorigenic group.

The patterns of tumor growth in these experiments and their cytological and histological characteristics were similar to those previously described (7). In all animals, the tumors displayed a highly invasive growth pattern. Tumors became palpable after 8–10 days. Twenty-eight days after the implantation, they were characterized by an important central necrotic core with many necrotic parts disseminated within the tumor. Macroscopic examination of tumor sections adjacent to the microdialysis probes revealed that the tip of the probes was at times located close to a necrotic area. Most of the membrane area available for exchange, however, was located in vascularized zones of the tumors.

Table I. Protein Levels (g/l) at Day 0 to Day 28 in Healthy and in Tumorigenic Rabbits

	Day 0		Day 28	
	Healthy	Tumor	Healthy	Tumor
Total proteins	59.5 ± 4.2	58.3 ± 4.4	58.5 ± 3.3	63.8 ± 3.8
Albumin	43.8 ± 3.5	41.5 ± 2.9	40.8 ± 3.4	29.1 ± 4.0*
α-globulins	4.8 ± 1.0	4.3 ± 0.6	4.7 ± 0.7	8.7 ± 1.6*
β-globulins	7.9 ± 0.6	7.8 ± 1.7	8.7 ± 1.4	14.3 ± 0.4*
γ-globulins	3.0 ± 0.6	4.7 ± 1.8	4.4 ± 0.3	11.7 ± 2.6*

Note: (n = 4; mean ± S.D.).
* Significantly different from mean at day 0 value ($P < 0.03$).

Plasma Pharmacokinetics

Total plasma concentration of MTX after i.v. administration exhibited a three exponential decay in both healthy and tumorigenic animals (Fig. 1 and 2, respectively). Peak plasma levels of total MTX were in the range of 350 to 770 μM in the control group and 470 to 680 μM in the tumorigenic group. There was no significant difference in the terminal half-life of total MTX in plasma between healthy and tumor-bearing rabbits (Table II). In contrast, a trend was noted toward a reduced volume of distribution and clearance of MTX in the tumorigenic group.

In healthy rabbits, the peak plasma level of unbound MTX was in the range of 265 to 570 μM. The concentration-time profile of free MTX was closely related to that of total MTX in plasma (Fig. 1) and the extent of plasma protein binding of MTX was 29 ± 9%. In the tumorigenic group, the peak plasma level of the unbound MTX was in the range of 152 to 490 μM. The concentration-time profile of unbound MTX declined in parallel with that of total MTX in plasma (Fig. 2), except for the very early time-course in one rabbit (T3). There was no significant difference in the terminal half-life of unbound MTX in plasma (table II) between healthy and tumor-bearing animals. The important variability of the plasma protein binding of MTX (33 ± 20%) was due to one rabbit (T3) which exhibited a very high plasma protein binding (69%). Excluding this animal, the plasma protein binding of MTX was estimated to 24 ± 3%.

Tissue Pharmacokinetics

In vitro recovery values reached a steady-state of 7.8 ± 1.5% within 30 min after the start of the probe perfusion. *In vivo* recovery at steady-state was close to that determined *in vitro:* 8.2 ± 4.1% and 7.7 ± 3.1% in muscle and tumor ECF, respectively (Fig. 3).

In healthy rabbits, the maximal MTX concentration in muscle ECF (C_{max} = 8.7 ± 3.4 μM) was reached 7.5 min (t_{max}) after drug administration and ranged from 10.8 to 38.8 μM (Fig. 1). The concentrations of MTX then declined and closely followed that of total and unbound MTX concentrations in plasma. Muscle ECF concentrations of MTX were considerably lower than in plasma as evidenced by the relative muscle tissue exposure to MTX ($AUC_{M\text{-}ECF}/AUC_{free}$: 14.2 ± 8.8% with extreme values ranging between 4.1 and 25.9%). Moreover, muscle ECF MTX concentrations and unbound plasma MTX concentrations were highly correlated (r: 0.967; extreme values: 0.920–0.998).

Tumor-bearing animals exhibited an important inter-individual variability in the tumor ECF MTX concentrations. Indeed, peak MTX concentrations ranged between 0.9 and 85.7 μM. The penetration of MTX in tumor also appeared to be somewhat slower than in muscle as evidenced by peak concentrations (C_{max} = 23.1 ± 23.4 μM) occurring later than in muscle

Table II. Pharmacokinetic Parameters of Total and Free MTX in Plasma

	Healthy rabbits		Tumorigenic rabbits	
	Total	Free	Total	Free
$t_{1/2\lambda 1}$ (min)	2.5 ± 1.3	3.19 ± 2.1	3.1 ± 1.2	4.1 ± 2.0
$t_{1/2\lambda 2}$ (min)	20.9 ± 8.8	20.6 ± 7.9	26.8 ± 7.3	22.1 ± 13.4
$t_{1/2\lambda Z}$ (min)	288 ± 85	248 ± 88	294 ± 155	213 ± 70
V_Z (l/kg)	4.0 ± 2.8	—	2.9 ± 2.2	—
CL (ml/min/kg)	8.9 ± 4.3	—	6.5 ± 4.2	—
AUC (mM·min/l)	27.9 ± 18.8	20.4 ± 15.7	38.8 ± 21.5	27.4 ± 19.8

Note: (n = 5; mean ± S.D.).

(t_{max} = 22.5 min). During the terminal phase, the concentration-time profile of MTX in tumor ECF followed the concentration-time profile of total and free MTX in plasma. The terminal half-life of MTX in muscle and tumor ECF was not significantly different (table III). However, AUCs for tumor ECF concentrations showed a trend toward slightly higher values than in muscle and the tumor exposure was subject to a very important inter-individual variability ($AUC_{T\text{-}ECF}/AUC_{free}$: 23.9 ± 15.9% with extreme values ranging between 0.6 and 45.2%). Except in one rabbit (T1), there was no correlation between MTX free plasma concentrations and MTX ECF tumor concentrations: r = 0.564 (range: 0.245–0.913).

Table III. Pharmacokinetic Parameters of Unbound MTX in Tissue ECF

	Muscle	Tumor
$t_{1/2\lambda 1}$ (min)	3.6 ± 2.5	9.0 ± 4.6
$t_{1/2\lambda 2}$ (min)	10.7 ± 6.8	21.2 ± 7.5
$t_{1/2\lambda Z}$ (min)	204 ± 185	248 ± 173
AUC (mM·min/l)	2.2 ± 0.9	5.7 ± 5.8

Note: (n = 5 ; mean ± S.D.).

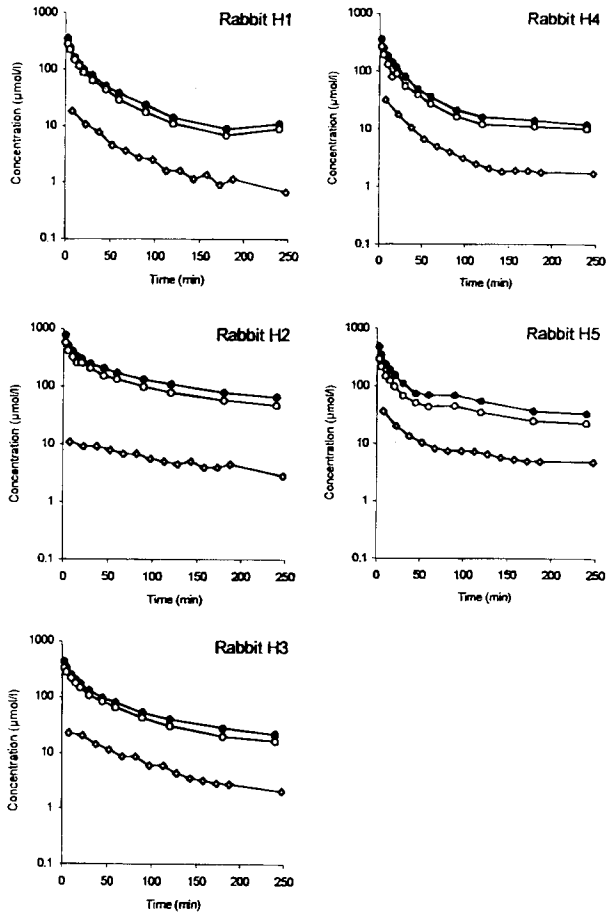

Fig. 1. Individual concentration-time profiles of total (●), free (○) MTX in plasma, and in muscle extracellular fluid (◇) in healthy rabbits after i.v. bolus injection of MTX (30 mg/kg).

DISCUSSION

The tumor model used in our study is an anaplastic squamous cell carcinoma derived from a domestic rabbit skin wart induced by the shope cottontail rabbit papillomavirus (8). The ease with which it can be maintained, the rapidity of its proliferation, and its size make it useful experimental material. Moreover, the size of the rabbit allows repeated blood sampling without extensive modification of the blood volume. Arterial sampling was carried out since peculiar and significant arteriovenous differences in MTX plasma concentration have been demonstrated after bolus administration of the drug in this species (9). Muscle was used as a model tissue in this study since MTX is known to show negligible conversion to its major metabolite (7-hydroxymethotrexate) in this tissue (10). Finally, the dose of MTX used in this study was chosen in order to provide plasma concentrations in the range of those measured in humans receiving high doses as commonly seen in the treatment of solid tumors (11,12).

The choice of a three compartment model was based on statistical considerations using the Akaike criterion. A mammillary three compartment open model with administration and elimination from the central compartment was arbitrarily chosen since there was no statistical method available to differentiate among the many different theoretical models.

The pharmacokinetic profile of MTX in plasma is characterized by an important inter-individual variability in both groups. In spite of the short duration of the experiment, the plasma pharmacokinetic parameters estimated in healthy rabbits were in reasonable agreement with those previously reported in the same species (9,13,14). The difference between the plasma protein binding of MTX calculated in our study and those

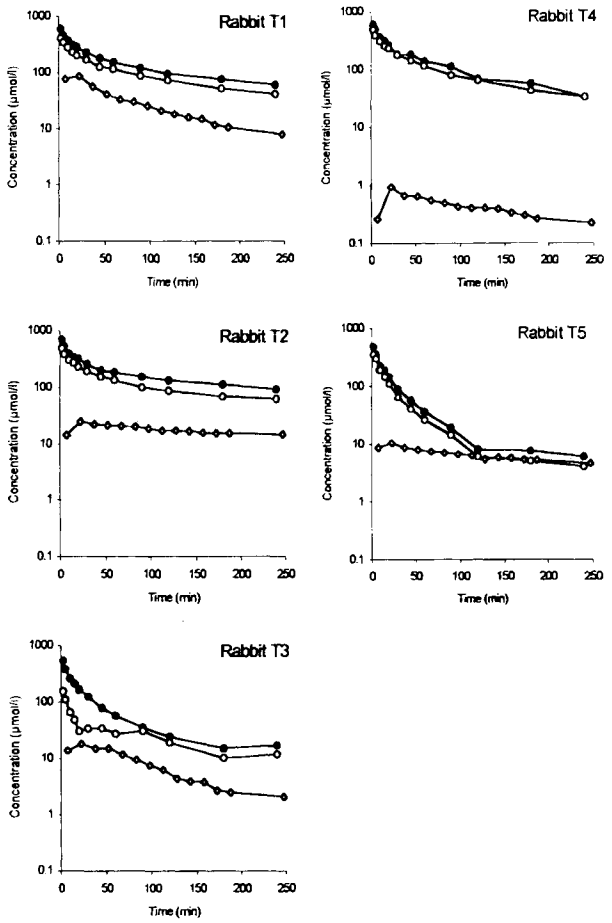

Fig. 2. Individual concentration-time profiles of total (●), free (○) MTX in plasma, and in tumor extracellular fluid (◇) in tumorigenic rabbits after i.v. bolus injection of MTX (30 mg/kg).

previously reported (56 ± 3.6% and 55 ± 6.4%) could be due to discrepancies in the experimental procedures used for its determination (14,15). In tumor bearing animals, the reduced total clearance and volume of distribution of MTX agree well with the significant decrease of both parameters previously reported in tumor bearing rats, and could be related to malnutrition and the presence of the tumor which is known to influence the host's metabolic state (16). Moreover, the trend toward a

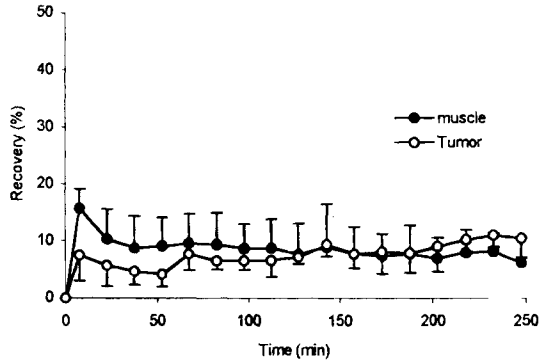

Fig. 3. *In vivo* recovery determination (mean ± S.D.) using the reverse dialysis method in muscle (●, n = 3) and in tumor tissue (○, n = 4).

reduced plasma protein binding of MTX is in agreement with the significant and positive correlation between MTX binding and albumin concentration as demonstrated in cancer patients (17).

The high perfusion rate used for microdialysis in this study was the result of a compromise between the volume of dialysate required for analysis and the sampling interval required to accurately establish the ECF concentration-time profile of MTX. The low recovery value is in the range of the values determined in different tissues of rats (4). This result was expected due to the known relationship between the perfusion rate and the recovery of MTX (4). The recovery was considered to be constant over the duration of the experiment since it has been reported that it is not concentration-dependent (2). This assumption could be validated by using a calibrator (internal standard) in the dialysate throughout the experiment to obtain more reliable estimates. The fact that *in vitro* and *in vivo* recoveries are very close may be surprising because *in vitro* recovery is not generally a reliable estimator of the *in vivo* recovery as reported for some endogenous compounds (urea, sucrose and radiolabeled water) or antipyrine using microdialysis in muscle (18). However, similarity between *in vitro* and *in vivo* recoveries is not unusual since it has also been demonstrated for some anticancer agents such as SR 4233 (19), a benzotriazine, MTX (17), and antibiotics such as piperacillin (20).

The low muscle ECF levels of MTX in the rabbit are in disagreement with the fairly similar muscle ECF and free plasma concentrations measured in the rat after i.v. injection of a 37.5 mg/kg dose of MTX (4). In contrast, they are in accordance with the poor distribution of MTX reported in the muscle of the rabbit (about 4% of the dose) and man (15,21). Finally, the very fast penetration of MTX in muscle ECF, the similarity of the concentration-time profile of the drug in muscle ECF and plasma, and the relatively low variability in muscle ECF levels of MTX justify the strong positive correlation between plasma and muscle ECF MTX levels.

Although the penetration of MTX is slower in tumor than in muscle ECF, the concentrations in tumor ECF are in the same range than that measured in muscle ECF. This result was expected due to the localization of the tumor and the site of implantation of microdialysis probes within the tumor. Indeed, our investigations were performed in a tumor implanted in the thigh muscle of the rabbit, a tissue known to exhibit a low exposition to MTX (15). It is also known that the blood flow within individual tumors varies considerably and is frequently lower in central and necrotic regions of medium and large size tumors which constitute the site of microdialysis. In addition and in contrast to most tumors, the VX2 tumor displays an average perfusion rate three times greater than muscle tissue, yet exhibits a similar extraction fraction (22). The terminal elimination half-life of MTX in tumor ECF was close to those estimated in plasma and muscle ECF. In contrast, the exposure of the tumor tissue to MTX is subject to an important inter-individual variability which could be partly explained by the heterogeneous nature of solid tumors which is subject to considerable inter-individual variation with regard to cellular organization. Similar findings were previously reported in rats bearing a brain-tumor (16) and could explain the absence of correlation between MTX blood concentrations and tumor ECF concentrations, except in one animal, and that any attempt to estimate of tumor ECF levels from plasma MTX concentrations would

be misleading under our experimental conditions. These results, in addition to the well known inter-individual pharmacodynamic variability may contribute to explain the fact that studies in patients with solid tumors have often failed to demonstrate strong correlations between MTX blood pharmacokinetics and chemotherapeutic response (1).

In conclusion, determination of ECF concentrations of MTX by microdialysis revealed a discrepancy between the exposure of the tumor tissue and the tissue of origin. More important, it has been established that there is a lack of correlation between ECF MTX concentrations in tumors at an advanced stage of development and plasma concentrations. This study emphasizes the advantages of such experimental models in understanding the targeting and disposition of anticancer drugs into the tumor, and thus, to establish more rationale and effective chemotherapeutic regimen in the treatment of tumors. Investigations using tumors at an earlier stage of development, and thus exhibiting different features than those seen in this study, are in warranted to assess whether the lack of correlation depends on the stage of development of the tumor.

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